Rational enzyme-directed prodrug development: exploiting tumour hypoxia to target the bioactivation of cytotoxic prodrugs

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RATIONAL ENZYME-DIRECTED PRODRUG DEVELOPMENT:

EXPLOITING TUMOUR HYPOXIA TO TARGET THE BIOACTIVATION OF CYTOTOXIC PRODRUGS.

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Dedicated to:

My wife Christina and our beautiful daughters Azura-Amy and Tallulah-May for their patience and understanding during the long days spent without them.

Also to my parents for their unwa vering support through the years of my education, and to my parents-in-law for rescuing my thesis in its hour of need!

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Abstract

Conventional cancer chemotherapy often lacks specificity and is consequently associated with significant normal tissue toxicities. Molecular chemotherapy offers the potential to target the activation of inert prodrugs by utilising tumour-specific catalytic enzymes to restrict cytotoxicity to neoplastic tissues. Appropriate expression of therapeutic enzymes can be achieved by exploiting the genetic distinctions that exist between tumour and normal tissues through the use of tissue- or disease-specific promoters. Alternatively, the unique physiological differences that arise in solid tumour masses as a consequence of the abnormal vascular architecture might also be exploited to achieve therapeutic selectivity. The most conspicuous of these differences is the presence of areas of low oxygen tension (hypoxia) arising through both diffusion and perfusion-limited oxygen availability. Hypoxia is an important cause of radioresistance and is a independent prognostic indicator for local recurrence, metastatic spread and overall survival. Evidence also implicates hypoxia in chemotherapeutic resistance and genetic instability, as well as the progression of and selection for an aggressive neoplastic phenotype.

Attempts to eliminate tumour hypoxia have met with some success, but the opportunity to utilise thisphenomenonfor therapeutic gain, through the exploitation of the unique reductive tissue environment have lead to the development of hypoxic-specific cytotoxins. These bioreductive prodrugs rely on the natural complement of tumour enzymes to catalyse their activation under low tissue oxygen tensions. Levels of these reductases are potentially heterogeneous and are often down-regulated in the neoplastic state. The artificial reintroduction of high levels of reductive enzyme expression may be of significant therapeutic value, particularly if expression is restricted to the hypoxic tissue enviroment in which the prodrugs will be activated. This might be achieved through the utilisation of the specific *cis*-acting sequences that are responsive to hypoxia-regulated transcription factors.

A diverse spectrum of genes are known to be induced as a consequence of oxygen deprivation, being involved in systemic oxygen supply, vascular tone, neovascularisation, iron homeostasis, glucose metabolism, drug detoxification and protein chaperoning. The details of the *cis*-acting sequences and transcription factors that mediate this oxygen-sensitive gene control are beginning to emerge. This provides the opportunity to exploit these defined sequences to regulate therapeutic genes in a hypoxia-responsive manner.

This thesis describes the evaluation of three potential prodrug/enzyme paradigms that may have application in this context. Further, the potential of hypoxia-response-elements to specifically regulate heterologous genes in response to low oxygen tension is described. The application of such an oxygen-regulated gene-directed enzyme/prodrug therapy to solid tumours may provide chemotherapeutic specificity aimed at a clinically important tumour subpopulation.

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Chapter 1

1. Hypoxia in solid tumours.

1.1 Tumour vascularisation

Human tumours are not highly angiogenic when they initially develop and can remain without vascularisation for months to years (Review: Folkman, 1990; 1995). The progression of a solid malignant growth is only possible if it can recruit a vascular network from the host, providing a blood supply to allow the exchange of nutrients and waste products. This requires the attenuation or loss of certain angiogenic suppressors eg. angiostatin, endostatin and thrombospondin 1 with the concurrent induction of a variety of angiogenic stimuli (Rastinejad *et al.*, 1989; Taraboletti *et al.*, 1990; Folkman, 1995). Establishing of a net balance of pro-angiogenic factors is critical to the provision of the angiogenic phenotype, and is essential for the progression of both primary and metastatic neoplastic tissues (Folkman, 1995). Critically, the degree of tumour angiogenesis is inversely related to patient survival (Review: Fox, 1997).

Multiple cell types that have been implicated in the primary and secondary roles of tumour angiogenesis including tumour cells (epithelial and endothelial origins), macrophages, mast cells, leucocytes, lymphocytes and platelets. These cells produce a variety of factors that are implicated as components of the angiogenesis process, including; vascular endothelial growth factor (VEGF) (Kech *et al.*, 1989), acidic / basic fibroblast growth factor (FGF) (Klagsbrun *et al.*, 1986; Kandel *et al.*, 1991; Slavin, 1995), platelet-derived growth factors-A and -B (PDGF) (Risau *et al.*, 1992), placental growth factor (PLGF) (Maglione *et al.*, 1993) platelet-derived endothelial cell growth factor (PD-ECGF) (Moghaddam *et al.*, 1995) and type II nitric oxide synthase (iNOS, NOS2) (Jenkins *et al.*, 1995). The appearance of microregional hypoxia (low oxygen tension) is thought to be a key stimulus in the initiation of neovascularisation, since it can induce cell-type dependent elevations of the above mentioned angiogenic factors; VEGF (Shweiki *et al.*, 1992; Plate *et al.*, 1992; Goldberg and Schneider, 1994; Levy *et al.*, 1995; Ikeda *et al.*, 1995), acidic/basic FGF (Kuwabara *et al.*, 1995), PDGF-A and -B (Kourembanas *et al.*, 1990; Plate *et al.*, 1992b; Kuwabara *et al.*, 1995; Gleadle *et al.*, 1995), PLGF (Gleadle *et al.*, 1995), endothelin (Kourembanas *et al.*, 1991; Li *et al.*, 1994), PD-ECGF (Griffith *et al.*, 1997) and iNOS genes (Pohl and Busse, 1989; Hwang *et al.*, 1994;

Xue *et al.*, 1994; Melillo *et al.*, 1995). Cytokines such as tumour necrosis factor α (TNFα) (Ghezzi *et al.*, 1991; Lim *et al.*, 1996), transforming growth factor β1 (TGF-β1) (Bicknell and Harris, 1991; Klempt *et al.*, 1992), interleukin-1α (IL-1α) (Shreeniwas *et al.*, 1992; Ghezzi *et al.*, 1991), interleukin-6 (IL-6) (Yan *et al.*, 1995) and interleukin-8 (IL-8) (Karakuram *et al.*, 1994) are also known to be induced by low oxygen tension, and can work cooperatively with these angiogenic factors. Another potential source of oxygen-dependent cytokine production in tumours can occur through various infiltrating lymphocyte populations (Sunderkotter *et al.*, 1994; Scannell *et al.*, 1993). For example, TNFα is secreted by tumour-infiltrating mononuclear cells in a hypoxia-dependent manner (Scannell *et al.*, 1993) which can in turn induce endothelial cells to produce bFGF. Mild hypoxia can marginally increase the expression of VEGF in vascular smooth muscle cells, but the co-treatment of mild hypoxia and either bFGF or TGF-β1 elicits a marked synergistic effect on VEGF production (Shweiki *et al.*, 1995). VEGF and bFGF in turn can synergise in the induction of angiogenesis (Pepper *et al.*, 1992). TGF-β1 stimulates angiogenesis *in vivo*, probably through induction of an inflammatory angiogenic infiltrate (Sunderkotter *et al.*, 1994), though it can also enhance angiogenesis by modulating the cellular production of extracellular matrix proteins (Kuzuya and Kinsella, 1994). IL-6 can promote vasorelaxation and the induction of proliferation of smooth muscle cells (Ohkawa *et al.*, 1994). The cytokines TNFα, IL-1α, bFGF, IFN-α, IFN-γ and perhaps TGF-β can each induce PD-ECGF and/or iNOS expression (Fox *et al.*, 1996; de Vera *et al.*, 1996). A number of angiogenic factors such as VEGF, iNOS, PD-ECGF are regulated by hypoxia in epithelium and other tissue types (Claffey *et al.*, 1992; Pohl and Busse, 1989; Hwang *et al.*, 1994; Fox *et al.*, 1996a; Griffith *et al.*, 1997), while tissue oxygenation affects the regulation of some cytokine growth factors specifically in endothelial cells, including PDGF-B, IL-1α, IL-8 and endothelin. The expression of cognate receptors for endothelin-1 and VEGF (Flt-1/Flk-1) (Shweiki *et al.*, 1992; Plate *et al.*, 1992a, 1993; Sandner *et al.*, 1997; Kourembanas *et al.*, 1991; Li *et al.*, 1994) are also upregulated on endothelial cells in response to oxygen deprivation, suggesting that tumour angiogenesis is regulated in a paracrine fashion (Plate *et al.*, 1992a). VEGF shares primary sequence homologies with other hypoxia-inducible cytokines; PLGF, PDGF-A and -B, of which PLGF has been shown to form functional heterodimers with VEGF (Disalvo *et al.*, 1995). A number of important adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), endothelial leucocyte adhesion molecule-1 (ELAM-1) and P-selectin are also upregulated on endothelium in response to oxygen deprivation (Goebeler *et al.*, 1993; Closse *et al.*, 1996). Of note, P-selectin is important in the initial step of neutrophil recruitment. Hypoxia dependent increases in protein expression can result from several mechanisms including; enhanced transcription, improved mRNA stability and extended protein half-life (Review: Bunn and Poyton, 1996). The molecular mechanisms

underlying the nature of these and other oxygen-responsive genes are discussed in detail in section 1.6.

As a malignant growth progresses, the preexisting host vessels can be incorporated into the tumour from which neovascularisation arises, fulfilling the microcirculatory needs within some areas of the expanding tissue mass. The influence of tumour angiogenesis factors will also promote neovascularisation from venules of the host tissue adjacent to the invasion front. Newly formed vascular sprouts grow out of the venular sites, fusing randomly to generate loops and ultimately anastomosing with arteriolar vessels. The static columns of red cells are then free to flow, and a microcirculatory network begins to establish. Yet, despite the aggressive process of neovascularisation, a Malthusian principle can operate, characterised by a more rapid increase of oxygen-consuming tumour cell populations than expansion of the oxygen-supplying functional microvasculature (Vaupel *et al.*, 1989a). Consequently a tumour mass will frequently outstrip the growth of the supporting vascular supply. This deficiency can be accentuated by the highly disorganised vascular architecture (Folkman, 1990), often with limited development of supporting collateral vessels bearing hastily formed microvessel structures; many of which show severe structural and functional abnormalities. Typically, vessel walls are characterised by interrupted or absent endothelial linings and basement membranes (Cater and Silver, 1960), as well as a lack of pericytes, contractile wall components and physiological receptors. Venules can develop marked contour irregularities, becoming tortuous and sinusoidal, with both exaggerated elongation and dilation, and display a significant loss of morphological heirarchy (Warren, 1979). Venular sprouts can fuse with other venules so that the newly formed vascular network is both supplied and drained by venules. This chaotic and spatially inadequate vascular architecture gives rise to large and variable intercapillary distances, which severely limits oxygen availability (Kolstad, 1968; Awwad *et al.*, 1986). The resulting oxygen tension (pO_2) gradients give rise to areas of chronic diffusion-limited hypoxia (Denger and Sutherland, 1988; Vaupel *et al.*, 1989a; Vaupel, 1990; 1993). Moreover, as a consequence of altered vascular morphology, tumour vessels are not only prone to spontaneous hemorrage and/or thrombosis (Gatenby *et al.*, 1988) but also to the invasion and/or compression of the vessel lumen by surrounding neoplastic tissues (Jain, 1988), As a result vessel integrity can be compromised and the blood supply can be temporarily slowed or halted, reducing oxygen availability even to the most favourably located tumour cell populations. Thus two forms of hypoxia are thought to exist in solid tumours: chronic hypoxia caused by limitations of oxygen diffusion, and acute hypoxia resulting from relatively rapid changes in tumour tissue perfusion.

1.2 Chronic hypoxia

It was first suggested by Thomlinson and Gray (1955), following a histological study of necrosis in bronchial carcinomas, that hypoxic cells exist beyond the diffusion distance of oxygen in solid tumours. They observed that all tumour cords of greater than 200 μm had a necrotic centre, while those tumour cords less than 160 μm showed no such evidence of necrosis. These histological observations in conjunction with some simplified oxygen diffusion calculations led Thomlinson and Gray to propose that tissue pO_2 would be effectively zero 150 - 200 μm from a functional capillary. It was suggested that along this limited diffusion distance would exist gradients of oxygen concentration, where cells could remain viable. Later studies modelling the oxygen supply and tissue oxygenation in tumours (Denger and Sutherland, 1988), as well as evaluation of oxygen diffusion distances in human xenograft models (Groebe and Vaupel, 1988) provided further evidence in support of the existence of chronic diffusion-limited hypoxia. For example, Groebe and Vaupel provided evidence that radiosensitivity might be less than 10% of maximum at intercapillary distances above 100 μm, and if intercapillary distances exceeded 140 μm, areas of radiobiological hypoxia extended right up to the arterial end of the microvessel. Direct inter-capillary distance measurements in cervix uterus carcinomas have also been shown to predict for the presence of tissue hypoxia (Awwad *et al.*, 1986; Kolstad, 1968). More recently, using videomicroscopic analysis of tumours transplanted into rat dorsal-flap window chambers, Dewhirst *et al.* (1996) have identified the presence of some vessel segments (9%) with detectable plasma flow but low or absent erythrocyte flux. They have suggested that such vessels might occur in human tumour tissues and would constitute an additional source of chronic hypoxia. Further, some cell subpopulations might also experience chronic hypoxia if disadvantageously located in situations of high oxygen consumption, where local tissue demand exceeded supply (Vaupel *et al.*, 1987).

1.3 Acute hypoxia.

Transient perfusion-limited hypoxia is thought to arise from the circulatory "chaos" that is generated as a consequence of abnormal vascular morphology, geometric resistance (governed by the vessel diameter), and viscous resistance which is determined predominantly by the rheological properties of the blood (Review: Jain, 1988). As a result of the altered morphology, serious functional disturbances of the microflow are inevitably manifest including; arteriovenous shunt perfusion, regurgitation and intermittent blood flow, and unstable speed and direction of

flux. Geometric resistance to blood flow (Sevick and Jain, 1989a; 1989b; Boucher and Jain, 1992) may occur as a result of mechanical obstruction by micro- and macro-thrombosis, erythrocyte sludging, transient plugging of microvessels by white blood cells (WBC) (Honess *et al.*, 1996), platelet aggregation or the invasion and/or compression of the vessel lumen by surrounding neoplastic tissues (Jain, 1988). Viscous resistance is influenced by many factors including; haemoconcentration, aggregation of red blood cells (RBC), cell deformability, plasma viscosity and plasma protein concentration. Decrease in the systemic haematocrit with tumour growth has been reported for both humans and animals, and is associated with the onset of anaemia (Dintenfass, 1982; Vaupel *et al.*, 1987). This can influence the fraction of hypoxic cells within a tumour mass (Hill *et al.*, 1971).

Significant increases in RBC aggregation and rigidity have been reported in melanoma patients (Dintenfass, 1982), lung and bowel cancers (Dintenfass and Forbes, 1973; Dintenfass, 1975), breast cancer (Riley, 1976) and in various carcinomas (Tietjen *et al.*, 1977). At low flow rates RBC will tend to form large aggregates (rouleaux) which raise blood viscosity and can potentially block flow through tumour microvessels. The bridging of adjacent RBC by fibrinogen, globulins and other macromolecules (eg. dextrans) also causes these cells to aggregate and form rouleaux. Increasedfibrinogenand albumin concentrations may also explain the elevated plasma viscosity. The increased viscosity will also reduce flow, markedly suppressing the shear rates in the blood. For example, in normal blood (45% RBC @ 37°C) a 10 -fold increase in viscosity can equate to a 1000 -fold drop in shear rate (Chein *et al.*, 1984a). The reasons for increases in RBC rigidity are less clear, although low pH and changes in plasma tonicity (hypo- or hypertonic) make RBC less deformable (Dintenfass, 1982; Chein *et al.*, 1984a). WBC rigidity can be three to five orders of magnitude higher than RBC rigidity (Chein *et al.*, 1984b), and circulating cancer cells even more so (Jain and Ward-Hartley, 1987). Thus a component of the increased viscous resistance in tumours might be attributed to increased WBC flux and the presence of intravascular tumour cells. Furthermore, in the presence of large RBC aggregates, WBC are forced towards the vessel wall, raising their local concentrations. This lateral displacement of WBC by RBC also occurs in vessels with increasing diameter. Additionally, in convergent flows, WBC entering from one capillary tend to be pushed to the wall by RBC entering from another. All these characteristics can be found in tumour postcapillary venules, since they are formed by converging capillaries which are gradually increasing in size, and can have the lowest shear rates in the circulation, thus favouring rouleaux formation. Consequently all these conditions collectively promote WBC migration to the vessel wall, so that ~ 95% of WBC roll slowly along the wall in postcapillary venules, greatly increasing opportunities for adhesion and abnormal platelet aggregation.

Tumour vasculature is also characterised by hyper-permeability and high hydraulic conductivity (Gerlowski and Jain, 1986; Sevick and Jain, 1991), which raises the microvascular hydrostatic pressure, and increases interstitial fluid pressure (IFP). IFP has been shown to be higher in tumours than in most normal tissues (Jain, 1987) and some evidence from experimental tumour models suggests that IFP may be inversely related to perfusion rates and oxygen tensions (Lee *et al.*, 1992; Roh *et al.*, 1991a). Data from clinical studies of patients with squamous cell carcinoma of the uterine cervix is consistent with these experimental studies (Roh *et al.*, 1991b; Milosevic *et al.*, 1995). This might be anticipated, since the actual blood flow rate through the tumour vasculature will depend to some extent upon the perfusion pressure and the vascular and interstitial resistances to that pressure. Sevick and Jain (1989a, 1989b) studied flow resistance under a variety of perfusion conditions using an isolated perfused tumour model, and demonstrated that flow resistance increased disproportionatly with reduced perfusion pressure. Both geometrical and viscous resistances contributed independently to the increase in flow resistance, with the increase in geometric resistance being indirectly related to collapse of vessels at low perfusion pressures as a result of elevated IFP. This hypothesis has been confirmed in both murine and human tumours (Boucher *et al.*, 1990, 1991; Roh *et al.*, 1991b).

However there is no direct reason to believe that IFP of tumours should be closely correlated to perfusion rate or pO_2, since each depends upon several biological, physiological and biophysical properties which are not all inter-dependent (Jain, 1987, 1988; Vaupel, 1990). Consistent with this, some studies have failed to find correlations between IFP and tumour pO_2 microelectrode determinations (Tufto *et al.*, 1996). However the pronounced bulk transfer of fluid in the interstitial space that accompanies the high interstitial fluid pressure can almost certainly cause progressive vascular compression. Moreover, venous pressures in tumours are significantly lower than in normal tissues (Peters *et al.*, 1980), and this poor vascular tone along with the relative fragility of tumour vessels will confer sensitivity to these external fluid and tissue pressures, resulting in periods of flow stasis and vascular collapse. These interruptions in blood flow generate transient foci of acute hypoxia in tumour tissues normally proximal to well established microvascular networks (Reinhold *et al.*, 1977; Brown, 1979).

Fluctuating blood flow in tumour microvessels has been recognised since the invention of the window chamber model (Algire, 1943; Algire and Chalkley, 1945; Endrich *et al.*, 1977, 1982; Intaglietta *et al.*, 1977). The therapeutic relevance of such intermittent perfusion has been demonstrated in transplantable murine tumours (Chaplin *et al.*, 1986) through its relationship with the induction of transient radiobiological hypoxia. Using cell sorting techniques in

conjunction with the fluorescent perfusion marker, Hoechst 33342, Chaplin *et al.* (1986) showed that if the DNA-binding dye was injected into mice 20 minutes before irradiation, there was no link between staining intensity of tumour cells and radioresistance. However when the dye and radiation were administered simultaneously, there was a direct relationship between stain intensity and radiosensitivity. Further work combining the Hoechst dye technique with fluorescent cadmium disulphide microparticle injection in the SCCVII murine carcinoma model, given together, or with a 20 minute time interval (Chaplin *et al.*, 1987; Trotter *et al.*, 1989), corroborated these initial observations. Comparable results have been reported in other murine tumour models (Jirtle, 1988; Minchinton *et al.*, 1990) and some human tumour xenografts (Chaplin and Trotter, 1990) indicating that perfusion-limited hypoxia is a common experimental phenomenon. Image analysis of SCCVII tumours following simultaneous or sequential fluorescent dye administration (Trotter *et al.*, 1991) identified regions of decreased staining intensity, as well as areas completely devoid of staining of one dye *vs.* the other. This incidence of total dye mismatch was 8%, which was similar to the levels of complete vascular stasis or intermittent blood flow subsequently reported by Dewhirst *et al.* (1992) in a transplantable mammary tumour model growing in a dorsal flap window chamber. Further, Trotter *et al.* (1991) found evidence of coordinated fluctuations in the staining intensity of contiguous vessel segments, and speculated that such vessels never reached total vascular stasis, but experienced periods of reduced flow in the time between administration of the first and second dye markers. They suggested that induction of hypoxia does not require total cessation of tumour blood flow.

In agreement, Dewhirst *et al.* (1996) found direct evidence correlating temporal changes in microvessel erythrocyte flux (but not necessarily total vascular stasis) with changes in the oxygen content of the same vessel. Utilising videomicroscopy, R3230AC tumours were transplanted into Fischer-344 rat dorsal-flap window chambers, and erythrocyte and plasma flux were visualised using 1% carbocyanine-labelled peripheral red cells and liposomes respectively, while perivascular oxygen concentration was monitored using recessed tip microelectrodes. In the unperturbed conditions of this model no evidence was seen for total vascular collapse, followed by reopening or reperfusion. Two main types of aberrant flow were observed. Firstly, marked instability of flow magnitude and direction occurred,as well as total vascular stasis. This was usually confined to a single vessel (4% incidence) and persisted for a few seconds at a time. Secondly, groups of vessels underwent coordinated cycles of fluctuations in flow, ranging from 20 - 60 minutes. Other microvessel segments were identified (9% of total) where plasma flow was evident, but very low or absent red cell flux was seen over many minutes. These complex patterns of restricted blood flow were associated with reductions in oxygen microelectrode readings. The pattern of tumour arteriolar vasomotion was also assessed, and coordinated

reductions in diameter could be seen between large arterioles and several daughter vessels. Dewhirst *et al.* (1996) speculated that the temporal characteristics of the oscillations were similar to the fluctuations in red cell flux (20 - 60 min) suggesting a cause and effect relationship. Theoretically the degree to which vasoconstriction affects the oxygenation of nearby tissues will depend upon the frequency of the oscillations (Secomb *et al.*, 1989). High frequency changes (≤ 1 Hz) are likely to influence the oxygen status of proximal cells, while the lower fequency oscillations will affect a greater tissue volume, with the rate of local oxygen consumption partially dictating the extent of the latter phenomenon.

Since the techniques employed in these experimental tumour systems are not directly applicable to the clinic, multichannel laser Doppler microprobes have been developed to directly monitor changes in microregional blood flow in both experimental tumour xenografts (Chaplin and Hill, 1995; Hill and Chaplin, 1995; Hill *et al.*, 1996) and human tumours in the clinic (Hill *et al.* 1996; Powell *et al.*, 1997). Using 300 μm diameter probes, fluctuations in erythrocyte flux can be recorded in tumour microregions with an estimated volume of 0.01 mm^3. Inital studies in an undifferentiated murine sarcoma SaF xenograft model (Chaplin and Hill, 1995) recorded changes of 2-fold or greater in 48% of microregions examined over a 60 minute period. Similar results (37%) were seen in the human colon adenocarcinoma HT-29 xenograft model (Hill *et al.*, 1996), and in both systems over 50% of the changes in blood flow occurred within 20 minutes. Clinical analysis of both primary and recurrent breast carcinomas, as well as metastases to regional lymph nodes and skin from a variety of tumours of different histologies, revealed similar results (Hill *et al.*, 1996). Of 66 human tumour microregions sampled, 26% showed a change in red cell flux by at least a factor of 2, while 58% of the samples demonstrated ≥ 1.5 - fold change. The frequency of slower, more persistent fluctuations was greater than that seen in the earlier experimental systems. However a high proportion of the changes measured still occurred within 20 minutes and in at least 30% of cases the change was reversed within the 60 minute observation period. A factor of 2 reduction in perfusion could correspond to a 50% reduction in blood flow in all the vessels contained in the probes' sampling volume, or at the other extreme, the complete closure of 50% of the vessels contained within the microregion. Likewise, a 1.5 -fold decrease would result if flow ceased in 30% of the sampled vessels. The development of laser Doppler microprobes has been crucial in the demonstration of the temporal nature of microregional fluctuations in tumour perfusion. While no system presently exists to measure temporal changes in tissue oxygenation directly, the observation that radiobiologically hypoxic cells can result from dynamic perfusion changes (Chaplin *et al.*, 1987) supports the inference that acute hypoxia, arising from changes in vascular flux, which can occur spatially

and temporally on both a micro- and macroscopic scale, is a major contributing factor to the presence of low pO_2 in human solid tumours.

However caution is required in interpreting the relation between changes in microscopic, macroscopic or global tumour perfusion and tissue pO_2 status, since tissue oxygenation is the resultant of both oxygen availability (i.e. blood flow x arterial O_2 concentration) and the actual respiration rate of the cells. Arterial O_2 concentration is mainly determined by the pO_2 of the arterial blood, the shape of the O_2 dissociation curve, and hemoglobin concentration (Thews and Vaupel, 1985). Release of O_2 from the RBC into tissues is largely dependent on the shape of the dissociation curve (which can be shifted through changes of 2,3-DPG, pH, pCO_2 etc.), the fluidity of RBC, and the pO_2 gradient between the blood and the surrounding tissue. Oxygen consumption rates of tumours *in vivo* are generally intermediate (2 - 35 $\mu l\ O_2\ g^{-1}\ min^{-1}$) between normal tissues with low metabolic rates (eg. resting skeletal muscle ~ 3 $\mu l\ O_2\ g^{-1}\ min^{-1}$) and normal tissues with high activities (eg. active skeletal muscle ~ 150 $\mu l\ O_2\ g^{-1}\ min^{-1}$) (Fleckenstein *et al.*, 1984; Lund, 1985; Vaupel, 1989a), although oxygen consumption will adapt to changes in availability.

Measurements of the HbO_2 saturation of individual RBC in experimental tumour microvessels have been achieved using cryospectrophotometric *ex vivo* techniques (Vaupel *et al.*, 1978). Characterisation of the oxyhemoglobin saturation status of murine tumours and xenografted human tumours (Rofstad *et al.*, 1988; Fenton *et al.*, 1988) demonstrates that HbO_2 saturations are significantly lower in tumours than in normal tissues. These HbO_2 values were gradually shifted downwards as the tumour volume increased. Furthermore, different tumour cell lines revealed different oxygenation patterns, and substantial intratumour heterogeneity and tumour-to-tumour variability was found (Vaupel and Mueller-Klieser, 1986; Vaupel, 1991). The *in situ* assessment of some human tumours has also been conducted, and the mean HbO_2 values observed in the tumours were markedly lower than those found in the surrounding normal tissues. For example, in differentiated adenocarcinomas of the rectum 72% of measurements showed HbO_2 saturation to be 60% or less, while in normal rectal mucosa only 7% of measurements were 60% or less, with no values below 45% (Figure 1) (Wendling *et al.*, 1984). Similar results were found between squamous cell carcinomas of the oral cavity and normal oral mucosa, with median of the HbO_2 frequency distributions of the normal oral mucosa being 80% compared to tumour medians of 49% saturation (Figure 2). Variations were shown to correlate with changes in vascular density (Mueller-Klieser *et al.*, 1981).

Figure 1.

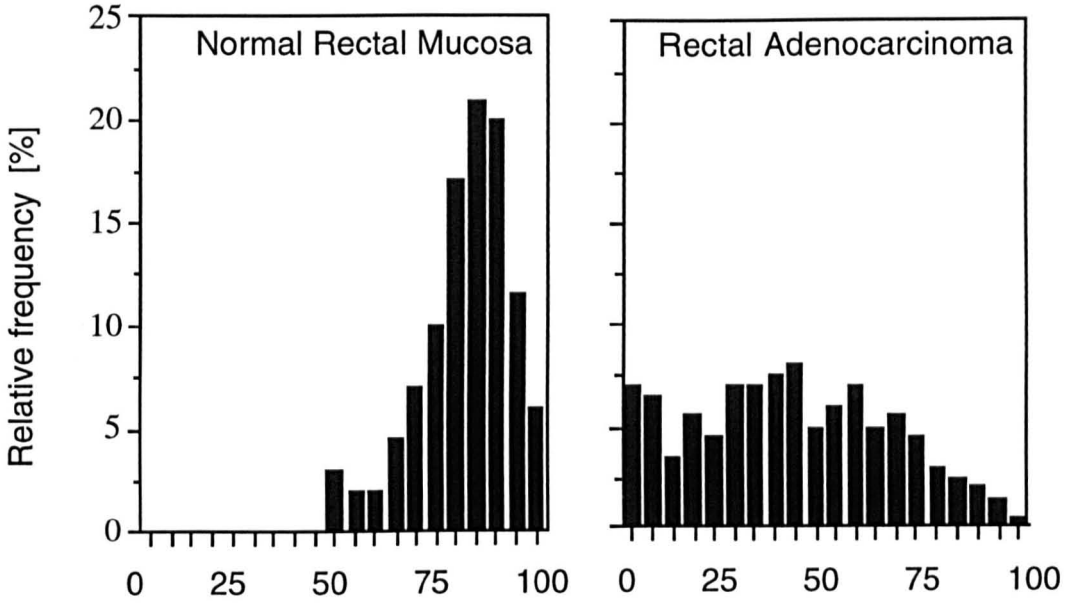
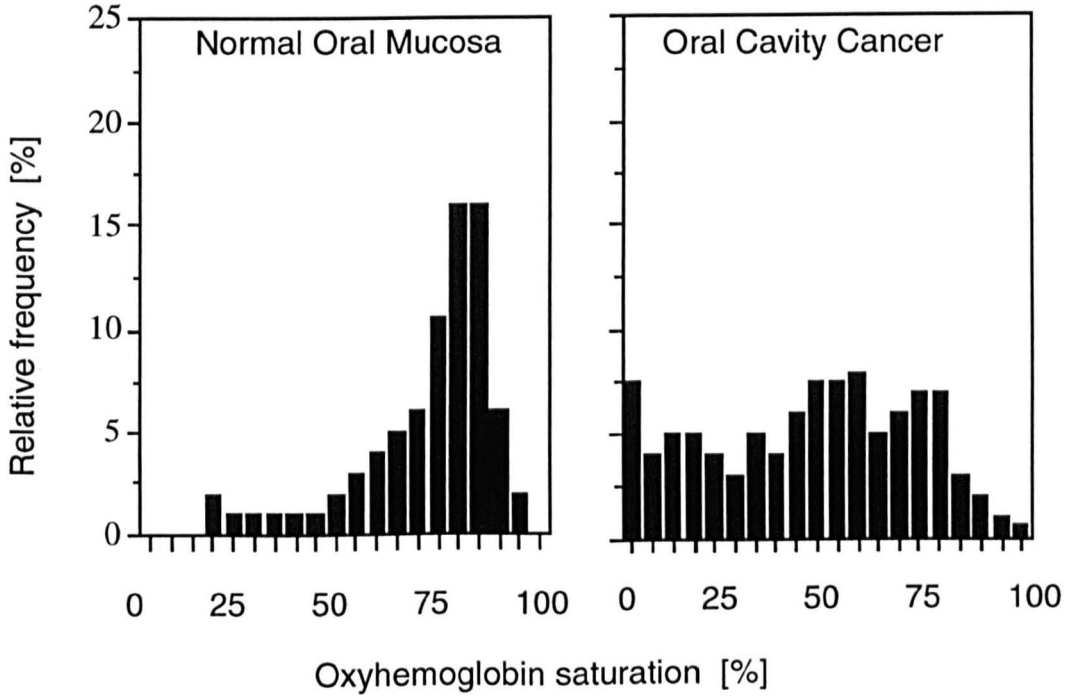


Figure 2.



Figures 1 and 2. Frequency distributions of measured oxyhemoglobin (HbO2) saturation values of individual red blood cells within microvessels of (1) normal rectal mucosa vs. differentiated rectal adenocarcinoma of the rectum, and normal oral mucosa and of poorly vascularised cancers of the oral cavity. Adapted from Wendling *et al.* (1984); Mueller-Klieser *et al.* (1981).

Considerable intra- and inter-tumoural differences were observed, even when tumours of the same clinical stage and histological grade were compared. Similar intra-individual variability was also found in a metastatic lesion of a lung cancer (Vaupel *et al.*, 1989a). Consideration should also be given to the fact that a proportion of global tumour perfusion does not result in the significant exchange of nutrients due to arteriovenous shunt flow. This arises from aberrant arterial-venous connections, where intermediate microcapillary structures are minimal or absent. Estimates of arteriovenous shunt perfusion in experimental tumours showed that at least 30% of the arterial blood can pass through the malignant tissue without taking part in the microcirculatory exchange process (Endrich *et al.*, 1982; Vaupel *et al.*, 1978; Weiss *et al.*, 1979). Shunt flow was reported to account for 8-43% (mean ± S.E. = 23±13) of total blood flow in patients receiving intraarterial chemotherapy for head and neck cancer (Wheeler *et al.*, 1986).

1.4 Direct determinations of tumour hypoxia

With the advent of the Eppendorf polarographic electrode system, direct measurements of tumour tissue oxygenation became possible, and the existence of regions of low pO_2 was demonstrated clinically (Kallinowski *et al.*, 1990; Vaupel *et al.*, 1991; Hockel *et al.*, 1993). While this technique provides a global picture of pO_2 distribution throughout a tumour, it does not reflect the dynamic changes in oxygen tension which may occur transiently in different regions of the tumour due to impaired perfusion or vessel closure. Furthermore, a major limitation of this electrochemical method of measuring oxygen in biological systems is that the measurement itself consumes oxygen, thereby perturbing the enviroment under study. In a heterologous enviroment and particularly at low oxygen levels, the measurement can be difficult to obtain and may not reflect the actual oxygenation state. However recent improvements in microelectrodes, including miniaturization and the addition of a recessed tip, have reduced both oxygen consumption rates and tissue volumes sampled (~12 μm diameter). In 1989 a computerised histography system was introduced, allowing rapid and reliable polarographic tissue pO_2 readings in the clinical setting (Kallinowski *et al.*, 1990; Vaupel *et al.*, 1991; Hockel *et al.*, 1991; Nordsmark *et al.*, 1994). These improved oxygen microelectrodes have also been used to demonstrate correlations between radiobiological hypoxia and direct estimates of tumour oxygenation both experimentally and clinically (Vaupel, 1977; 1979; Vaupel *et al.*, 1989a; Moulder and Rockwell, 1984; Kallinowski *et al.*, 1989; Gatenby *et al.*, 1985; 1988; Okunieff *et al.*, 1993; Horseman *et al.*, 1993, 1994), and has lead to a resurgence in their clinical application, leading to their designation as "gold standard" for measuring tumour oxygenation

(Stone *et al.*, 1993). Thus, despite the disadvantages, the Eppendorf oxygen probe is currently the system of choice in the clinical assessment of tumour hypoxia.

In studies, direct measurement of pO_2 in human tumours shows a wide variation in median oxygen concentrations, ranging from 1.1 to 3.9 %, (Vaupel *et al.*, 1989a, Vaupel, 1993; Gatenby *et al.*, 1985; 1988; Kallinowski *et al.*, 1990; Vaupel *et al.*, 1991; Hockel *et al.*, 1993; Rampling *et al.*, 1994; Nordsmark *et al.*, 1994; Brizel *et al.*, 1995). In contrast, the median pO_2 values of normal tissues range from 3.1 to 8.7 %, (Vaupel *et al.*, 1977; Cater and Silver, 1960; Ernst *et al.*, 1976; Gatenby *et al.*, 1985, 1988; Jamieson *et al.*, 1965; Wiess and Fleckenstein, 1986; Kolstad, 1968; Fleckenstein *et al.*, 1984; Lund, 1986; Wiener *et al.*, 1982; Cooper *et al.*, 1966; Roberts and Owens, 1972; Silver, 1979). Furthermore, readings of less than 0.3 % O_2 (2.5 mm Hg) are common, being found in up to 82 % of samples taken (Review: Vaupel, 1993). When the mean pO_2 values found in human malignancies are compared against their respective normal tissues, differences can be consistently demonstrated. These and other data are summarised in table 1 and are expressed as a ratio of mean pO_2 for normal *vs.* tumour tissue. Comparative examples for colon adenocarcinomas, glioblastomas, breast and cervix cancers and their respective normal tissue pO_2 distributions are shown in figures 3-6 respectively.

The phenomenon is independent of either staging and/or grading of individual tumours, as well as the tissue type (Figure 7-8). Such lack of predictability arises as a consequence of pronounced tumour-to-tumour variability, even amongst tumours of the same pathological stage and histological grade (Figure 9). Marked intratumour heterogeneity can also be seen, and examples of different oxygen partial pressure histographs being derived from consecutive needle tracks have been reported (Figure 10) (Vaupel, 1993).

However, Hockel *et al.* (1996) demonstrated that a minimal two track procedure was adequately representative of the median tumour pO_2 distribution, negating the requirement for multiple invasive determinations. Further, using the standard two track procedure, the inter-observer variation of the median pO_2, with respect to histograph, operator and time of measurement was of the magnitude 2-3 mm Hg. Notably, intratumour variation was similar irrespective of whether measuring positions were within a few millimeters of each other or spanned several centimeters. The observed tumour-to-tumour heterogeneity was significantly greater than that of the intratumoural variation, enabling this standardised procedure to categorise individual cervical cancers. Importantly, use of the needle oxygen probe was well tolerated (with only minor discomfort reported in a few cases). This direct approach to the

measurement of oxygen concentration adds convincing support for the presence of large differences in pO_2 between tumour and normal tissues.

Table 1: Ratio of mean pO_2 values found in human malignancies are compared against their respective normal tissues.

Tumour type	Ratio tissue mean pO_2 Normal : Tumour	References.
Cervix cancer		
Stage 0	1 : 0.63	Kolstad (1968)
Stage 1	1 : 0.42	Kolstad (1968)
Stage 2	1 : 0.31	Kolstad (1968)
Stage 2	1 : 0.56	Bergsjo & Evans (1971)
Undefined	1 : 0.29	Brizel *et al.* (1995)
Squamous cell cancers	1 : 0.42	Urbach and Noell (1958)
	1 : 0.40	Cater and Silver (1960)
	1 : 0.23	Gatenby *et al.* (1985)
	1 : 0.16	Urbach (1956)
Breast cancer	1 : 0.50	Badib & Webster (1969)
	1 : 0.43	Vaupel (1994)
	1 : 0.42	Cater and Silver (1960)
	1 : 0.23	Urbach (1956)
Melanomas	1 : 0.16	Gatenby *et al.* (1985)
	1 : 0.15	Urbach & Noell (1958)
	1 : 0.16	Urbach (1956)
Soft tissue sarcomas	1 : 0.36	Urbach (1956)
	1 : 0.16	Gatenby *et al.* (1985)
Malignant lymphomas	1 : 0.67	Badib & Webster (1969)
	1 : 0.45	Urbach & Noell (1958)
Adenocarcinomas	1: 0.14	Gatenby *et al.* (1985)
Basal cell epitheliomas	1 : 0.18	Urbach (1956)
Glioblastomas	1 : 0.31	Rampling *et al.* (1994).

Figure 3.

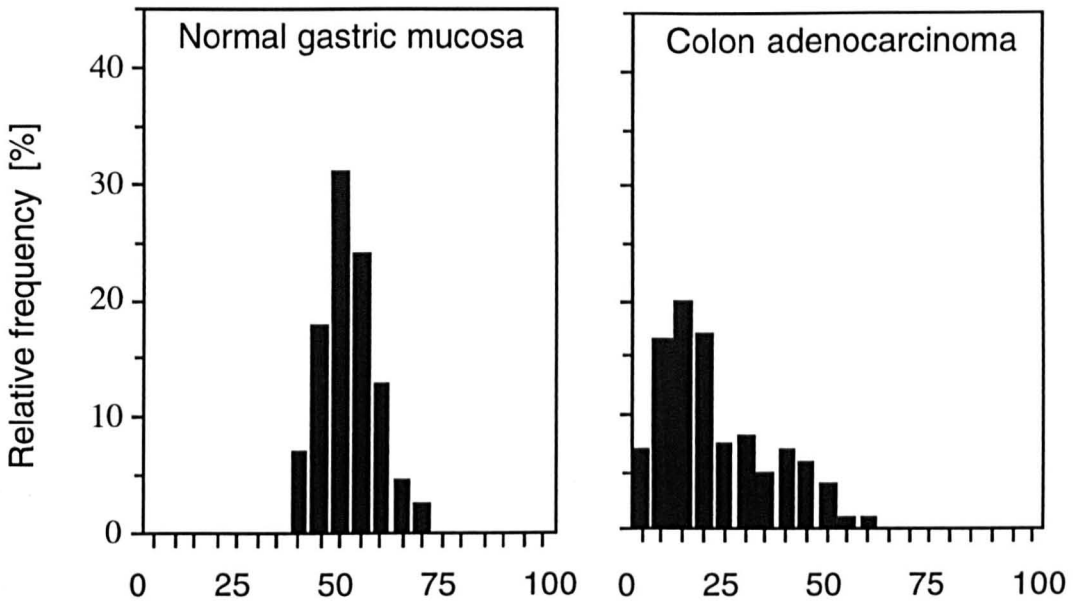
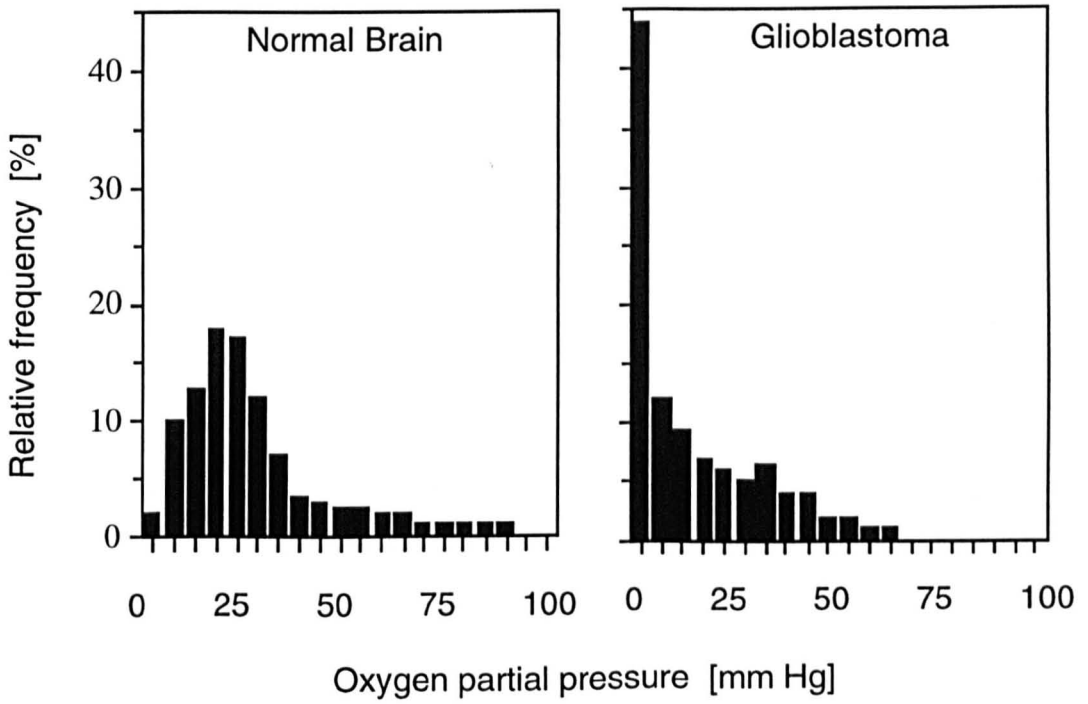


Figure 4.



Figures 3 and 4. Frequency distributions of measured oxygen partial pressures (pO_2 histograms) for normal vs. malignant tissues of the colon and brain respectively. Pooled data from various sources: Normal gastric mucosa, Endrich (1988); Colon adenocarcinoma, Gatenby *et al.* (1985) and Endrich (1988); Normal brain, Cooper *et al.* (1966), Roberts and Owens (1972) and Silver (1979); Glioblastoma, Rampling *et al.* (1994).

Figure 5.

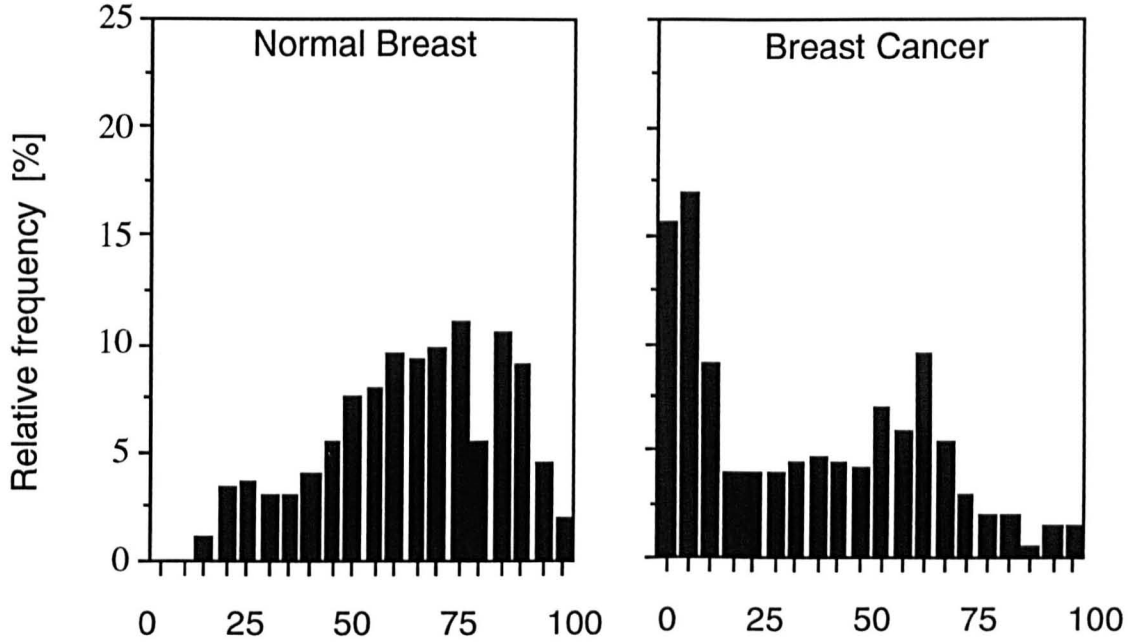
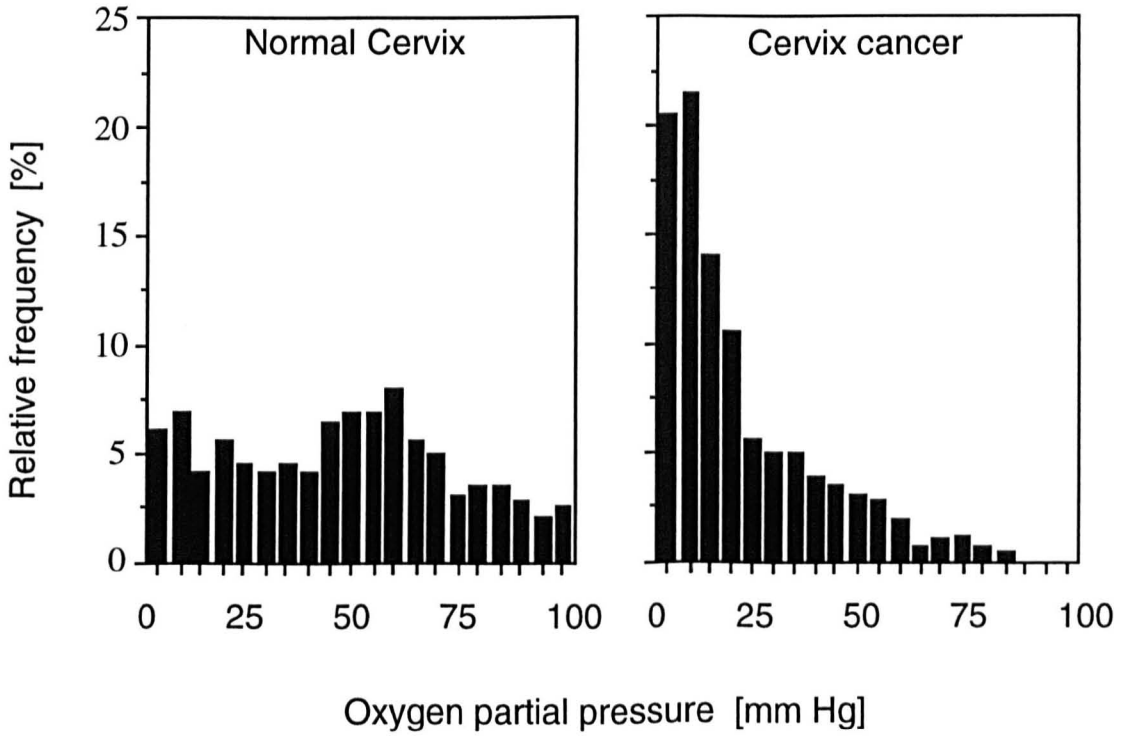


Figure 6.



Figures 5 and 6. Frequency distributions of measured oxygen partial pressures (pO_2 histograms) for normal vs. malignant tissues of the breast and cervix respectively. Pooled data from various sources: Breast, Hoeckel *et al.* (1992); cervix, Schlenger *et al.* (1991).

Figure 7.

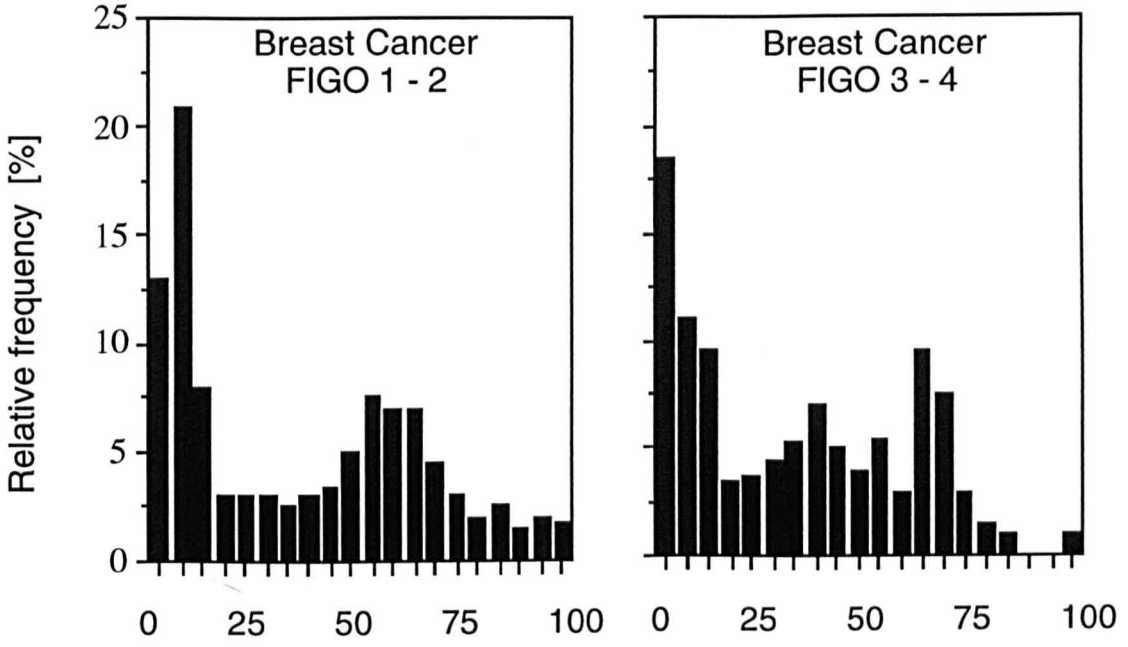
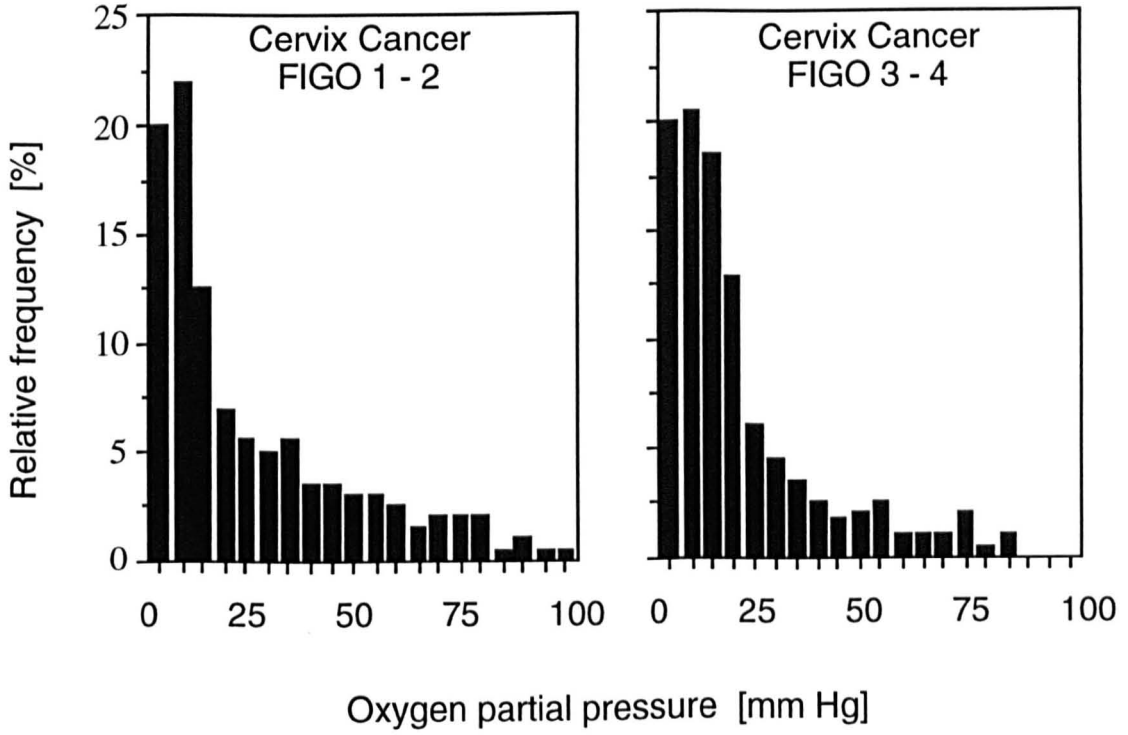


Figure 8.



Figures 7 and 8. Frequency distributions of measured oxygen partial pressures (pO_2 histograms) for malignant tissues of the breast and cervix respectively, at either stage 1-2 or 3-4. Dataadapted from various sources: Breast, Hoeckel *et al.* (1992); cervix, Schlenger *et al.* (1991).

Figure 9.

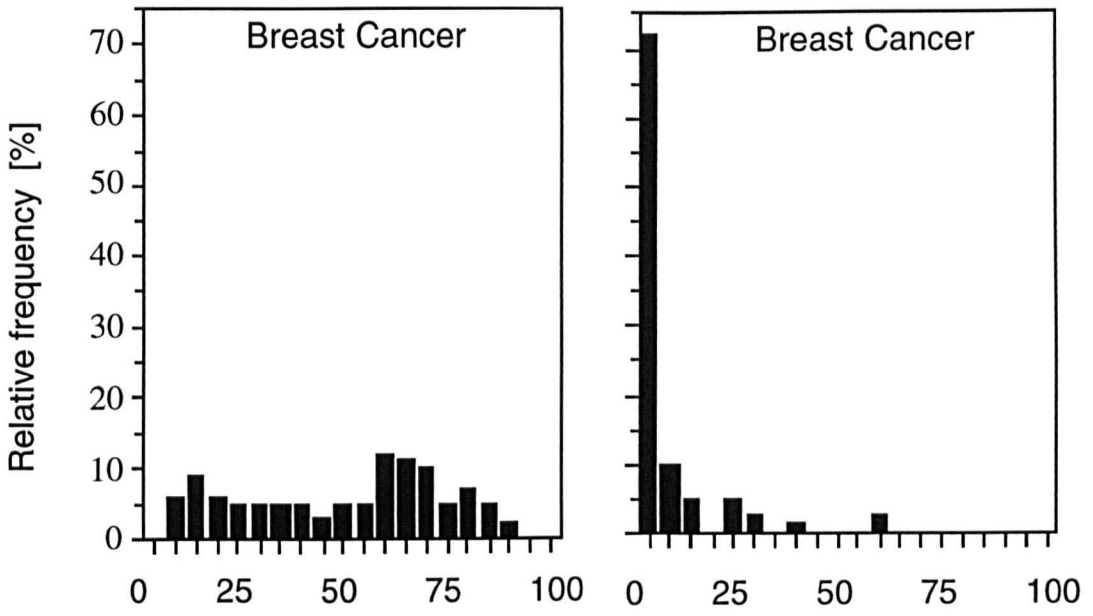
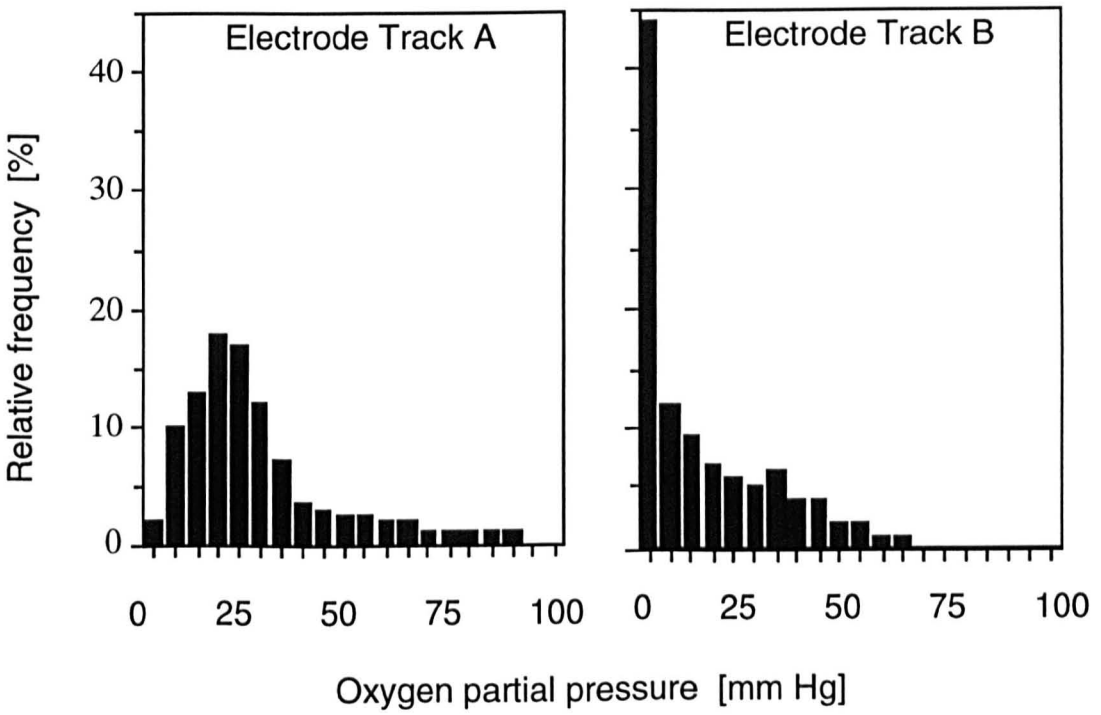


Figure 10.



Figures 9 and 10. Frequency distributions of measured oxygen partial pressures (pO_2 histograms), exemplifying marked tumour heterogeneity between (Fig.9) and within breast tumour tissues. Data from Vaupel *et al.* (1991); Vaupel (1990).

1.5 Alternative techniques for measurments of tumour oxygen status

Many other techniques, both direct and indirect, have been and are being developed to measure tumour tissue oxygenation status. (Reviews: Raleigh *et al.*, 1996; Stone *et al.*, 1993). The wealth of new and emerging imaging agents and techniques reflects the growing awareness of the importance of hypoxia in the etiology and management of human neoplasia.

Direct determination of radiobiological hypoxia can be achieved on an individual cell basis using the alkaline comet assay (Review: Fairbairn *et al.*, 1995; Olive and Durand, 1992; Olive *et al.*, 1990; 1993; 1994; Hu *et al.*, 1995). This assay relies on the 3-fold reduction in the number of radiation-induced DNA single strand breaks under radiobiologically hypoxic conditions (Olive *et al.*, 1993; Zhang *et al.*, 1995), the presence of which can be visualised following single cell gel electrophoresis of tumour fine needle aspirates. The cells are irradiated prior to being embedded and lysed in agarose gel. Since broken cellular DNA migrates more readily in an electric field than undamaged DNA, the greater the radiation damage the more the DNA will migrate out of the nucleus (Olive *et al.*, 1990). While it is the most direct measure of the radiation damage believed to underlie radiation response in tumour cells, estimations of experimental tumour hypoxic fraction do not consistently agree with other established techniques. For example, while Olive and Durand (1992) found good agreement between techniques, Hu *et al.* (1995) found that the hypoxic fractions detected using the comet assay were lower (by a factor of 3 to 6) than those measured by the paired survival method, an established radiobiological techinque to calculate the actual fraction of radiobiologically hypoxic cells. In the conventional paired survival method the hypoxic fraction is calculated by comparing the surviving fraction of tumour cells from air-breathing mice with those obtained from mice killed by carbon monoxide inhalation 5 min before irradiation. Thus the paired survival assay measures the hypoxic fractions of the clonogenically viable tumour cells, whereas the comet assay measures the hypoxic fraction of all cells in the tumour. Other confounding factors may arise from the forces involved in aspiration (i.e. background DNA damage) and sample contamination by unirradiated white blood cells (Hu *et al.*, 1995). The invasive nature of this technique is a disadvantage, and moreover sampling is subject to errors, and necessitates a relatively large single dose of radiation (minimum of 3.5 Gy). Also the repair half-time for the DNA single-strand breaks is very short ($t_{½} \approx 3$ min), requiring very rapid sample collection and processing (Wheeler *et al.*, 1992).

Chemical hypoxia markers are dominated by the 2-nitroimidazole (2-NI) compounds which are activated and bound to macromolecules within hypoxic cells with a dependence fortuitously close to that required for the radiobiological oxygen effect (Chapman *et al.*, 1981; 1983; Review: Chapman, 1991). Methods to visualise tissue-bound nitroimidazoles differ by nature of the detectable label and include; ^{19}F-hexafluoromisonidazole magnetic resonance spectroscopy and imaging (MRS and MRI) (Li *et al.*, 1991; Kwock *et al.*, 1992), ^{18}F-fluoromisonidazole positron emission tomography (PET) (Koh *et al.*, 1995), ^{99m}Tc-nitroimidazole scintigraphy (Linder *et al.*, 1994), ^{123}I-nitroimidazole single photon emission computed tomography (SPECT) (Groshar *et al.*, 1993), ^{3}H and ^{14}C-misonidazole autoradiography (Urtasun *et al.*, 1986; Chapman *et al.*, 1989) or immunohistochemistry (formalin-fixed tissue sections, FACS, ELISA) (Cline *et al.*, 1994; Lord *et al.*, 1993; Raleigh *et al.*, 1996). However limited concentrations of bound adducts and the relatively low detection sensitivity of ^{19}F MR can restrict clinical application (Workman *et al.*, 1992). Further, bioactivation of these agents to selectively bind in hypoxic tissues is dependent upon the complement of cellular reductases, which are known to vary across tumours (Chapman *et al.*, 1983; Workman *et al.*, 1992; Joseph *et al.*, 1994; Workman and Stratford, 1993). Joseph *et al.* (1994) demonstrated that both DT-diaphorase and xanthine oxidase were inefficent enzymes for reducing the 2-NIs, misonidazole and desmethylmisonidazole, while NADPH:cytochrome P450 reductase initiated metabolism was the major pathway for determining the 2-NI adduction to both aerobic and hypoxic COS-1 cells. Over 90% of the hypoxic binding reaction was inhibited by oxygen, suggesting that the one-electron reduction produces radical anions that are efficiently back-oxidised in the presence of molecular oxygen. The kinetics of binding to cells under hypoxic conditions was ~ ½ order with respect to P450 reductase, indicating the involvement of other enzymes in the further reduction of 2-NI to the nitroso and hydroxylamine derivatives. This is consistent with studies on the rat liver microsomal metabolism of benznidazole, a readily monitored model drug (Walton and Workman, 1987), where P450 reductase was shown to dominate the early stages of metabolism, particularly the one-electron reduction to the nitro radical. The cytochrome P450 isozymes were found to be more actively involved at the terminal phase of amine formation. The levels of P450 reductase and cytochrome P450 isoforms have been shown to be heterogenous in tumours (Stout and Becker, 1986; Hall *et al.*, 1989; Forrester *et al.*, 1990; de Waziers *et al.*, 1991; Shepard *et al.*, 1992; Philip *et al.*, 1994; Patterson *et al.*, 1997), with examples of tumour activities both higher and lower than that found in normal tissues.

Raleigh *et al.* (1996) demonstrated the discrepancies that can arise between 2-NI labeling techniques and direct Eppendorf oxygen electrode measurements. Using an *in vivo* rat mammary

carcinoma model they directly compared the O_2 tension variations along single tracks through the solid tumour with the observed radiobiological hypoxia, as defined by immunohistochemical analysis of the pimonidazole-labelled photomicrographs of the corresponding tissue sections. Clear examples of microregional zones of pimonidazole binding could be seen at points adjacent to the electrode track where pO_2 values (12 mm Hg) were apparently well above the K_m for both radiobiological hypoxia and pimonidazole binding. Raleigh *et al.* (1996) reported the presence of similar microscopic regions of pimonidazole binding in human cervical tumours, suggesting that hypoxic cells might exist in cells that are not accurately detected by oxygen microelectrodes. Kavanagh *et al.* (1996) have extended these studies in five transplantable murine tumours, comparing Eppendorf pO_2 histographs and [^{3}H]-misonidazole binding profiles with the paired survival assay to calculate the actual fraction of radiobiologically hypoxic cells. A correlation was observed between the mean values of the hypoxic proportion as measured by the paired survival assay and the mean binding of [^{3}H]-misonidazole ($r=0.94$; $P=0.02$), However no biologically significant correlation was seen between the mean values of the hypoxic cell fraction (paired survival assay) and the pooled Eppendorf pO_2 histograph measurements. Furthermore, when these two techiques were applied on the same individual KHT-C tumours, there was again no correlation between the two measurements of hypoxia.

Alternative approaches to ^{19}F imaging of tumour oxygenation have been developed including the use of perfluorocarbon probes (Rockwell, 1985), which are lipid emulsions of ^{19}F-substituted hydrocarbon molecules that can themselves carry high levels of dissolved oxygen. These suspended micelles are smaller than erythrocytes and when administered intravenously can not only enhance tumour oxygenation to assist radiotherapy (Teicher, 1992), but can also be employed to image vessels in a tumour mass (Sotak *et al.*, 1993). Since the MR signal characteristics are modified by O_2 concentration, image projections through tumours can be used to profile the tissue pO_2 distribution (Dardzinski and Sotak, 1994).

Non-invasive nuclear magnetic resonance techniques, relying on the abundance of natural endogenous NMR nuclides such as the proton (^{1}H) nucleus and phosphorous (^{31}P), can also be used to indirectly measure tissue oxygenation. ^{31}P MRS can monitor tissue bioenergetic parameters such as ATP, inorganic phosphate (Pi),phosphocreatine(PCr) and pH, all of which depend upon tissue oxygenation. The Pi/PCr ratio is considered useful in that it reflects changes in cellular [ADP] which are inversely dependent upon the tissue pO_2 requirements for ATP production *via* oxidative phosphorylation. Determinations of these parameters have been shown to correlate with O_2 electrode measurements (Vaupel *et al.*, 1989b; 1994), intracapillary oxyhaemoglobin saturation (Rofstad *et al.*, 1988a), $^{15}O_2$ perfusion measurements (Evelhoch *et*

al., 1986) and tumour fraction of radiobiological hypoxic cells (Rofstad *et al.*, 1988b; Sostman *et al.*, 1991). ^{1}H MRS can detect elevated lactate levels, which are associated with highly glycolytic tissues such as tumours, although the correlation between tissue lactate and pO_2 is questionable since many other factors such as glucose availability will be involved (Howe *et al.*, 1993; Vaupal *et al.*, 1994; Helmlinger *et al.*, 1997). However, of clinical relevance, high lactate levels in human cervical cancer have been shown to correlate with incidence of metastasis (Schwickert *et al.*, 1995). ^{1}H MRS can also be used to determine the tissue equilibrium of oxymyoglogin and deoxymyoglobin, although this technique is limited by low concentrations of myglobin in tumours (Shungu *et al.*, 1992). In contrast the application of ^{1}H nuclear magnetic resonance to direct tumour imaging, rather than spectroscopic analysis, has proved more useful. ^{1}H MRI can visualise changes in blood flow and oxygenation in some detail, since haemoglobin (like myoglobin) is paramagnetic, and hence the magnetic field around blood vessels is perturbed. Some MRI methods can exploit this characteristic (eg. gradient recalled echo sequences) and are very sensitive to the blood oxygen level dependent effect (Haase *et al.*, 1986). As a result this technique is sensitive to local variations in blood vessels, such as changes in haemoglogin concentration or the degree of O_2 saturation in the blood (Kwock *et al.*, 1992; Howe *et al.*, 1996). These methods are summarised in table 2.

Table 2: Summary of physical and chemical techniques for monitoring tissue hypoxia.

Technique	Invasive	Parameter measured	Reference
Polographic electrode - needle / fibre	Yes	extracellular pO_2	Kallinowski (1990) Stohrer (1992)
Polograph electrode - surface	Yes	extracellular pO_2	Kessler (1981) Fleckenstein (1984)
Oxygen-quenched luminescence	Yes	extracellular pO_2	Peterson (1984) Young (1996)
Phosphorescence	No	extracellular pO_2	Wilson (1996)
Bioreductive hypoxic markers	Yes	nitroimidazole binding	Hodgkiss (1991) Evans (1995)
Intrinsic markers	Yes	eg. LDH, VEGF	Murphy (1991)
^{31}P MRS	No	phosphate metabolites	Chance (1986) Vaupel (1989)
^{1}H MRS (lactate)	No	water protons	Stone (1993)
^{1}H GRE MRI	No	water protons - influenced by HbO_2	Robinson (1995)
^{19}F MRI	No	fluorinated bioreductives, perfluorocarbon emulsions	Rockwell (1991) Mason (1994)
^{19}F PET	No	fluorinated bioreductives	Koh (1992) Groshar (1993)
Near-infrared spectroscopy	No	deoxyhaemoglobin, oxyhaemoglobin, cytochrome aa3	Delpy (1987) Wahr (1996)
[^{123}I] IAZA	No	misonidazole analogue binding	Parliment (1992)
ESR	No	pO_2-paramagnetic probe molecules	Swartz (1992) Halpern (1996)
Cryospectrophotometry	Yes	oxyhaemoglobin saturation	Rofstad (1989) Mueller-Klieser (1990)
Bioluminescence imaging	Yes	ATP	Mueller-Klieser (1990)
HPLC of reductive metabolites	Yes	nitroaryl fluorescence	Stratford (1984)
Comet assay	Yes	DNA strand breaks - radiosensitivity	Olive and Durand (1992)
Alkaline elution assay	Yes	DNA strand breaks - radiosensitivity	Zhang and Wheeler (1993)
Tumour vascularity	Yes	vascular density	Awwad (1988)
Lactate	Yes	lactate production	Vaupel (1994)
Tumour interstitial pressure	Yes	interstitial pressure	Roh (1991)
Blood flow - laser Doppler flowmetry	Yes	red cell flux	Chaplin and Hill (1995); Hill (1996)

1.6 Oxygen sensing and the cellular response to hypoxia

Molecular oxygen is a chemically versatile, abundant, and permeating molecule. Its capacity to accept electrons allows dioxygen to participate efficiently in oxidation-reduction reactions necessary to release the metabolic energy of reduced organic chemicals. Eucaryotes (in contrast to procaryotes) have one major type of energy-conserving respiratory chain, located in mitochondria, and use oxygen as the terminal oxidant. Thus oxygen tension influences whether ATP will be produced primarily by aerobic respiration linked phosphorylation, or by catabolic reactions that generate ATP at the substrate level (Harold, 1986). Oxidative phosphorylation is far more efficient than substrate level phosphorylation in capturing the redox energy of reduced substrates (Krebs and Kornberg, 1957), with the oxygen-dependent tricarboxcylic (Krebs) cycle generating an 18-fold greater equivalent yield of ATP than the anaerobic glycolytic (Embden-Meyerhof) pathway. Utilisation of the primary cellular nutrients, glucose and fatty acids, to provide pyruvate for subsequent processing by oxidative phosphorylation, is far more energetically favourable than the generation of lactate by anaerobic glycolysis. Thus oxygen deprivation in solid tumour tissues impacts upon the pathways of glucose utilisation and so will elicit an adaptive physiological response at the cellular level.

A rapid increase in glucose utilisation occurs upon inhibition of mitochondrial function (Pasteur effect), the rate of which is determined by the transport of glucose into cells, and the activities of the three rate-limiting glycolytic enzymes; hexokinase, phosphofructokinase and pyruvate kinase. These enzymes are normally far from equilibrium and present in low abundance and their immediate regulation depends upon allosteric alterations of catalytic activity by substrate, product, nucleotides and redox state (Bunn and Poyton, 1996). More long term adaption also occurs with increases in biosynthetic rate of the genes encoding enzymes responsible for both high-capacity facilitated glucose transport, (primarily Glut-1 and -3) (Mueckler, 1994; Pessin and Bell, 1992), and glycolytic metabolism including; phosphofructokinase-L and -M (PFK-L and -M), pyruvate kinase-M (PK-M), aldolase-A and -C (ADL-A and -C), phosphoglycerate kinase-1 (PGK-1), enolase-1 (ENO-1), and lactate dehydrogenase A (LDH-A) (Firth *et al.*, 1994; Semenza *et al.*, 1994; Ebert *et al.*, 1995a, 1995b; Semenza *et al.*, 1996). The induction of glycolytic enzymes in response to oxygen deprivation is isozyme specific, reflecting the specific catalytic funtions of various isoforms (Ebert *et al.*, 1995b).

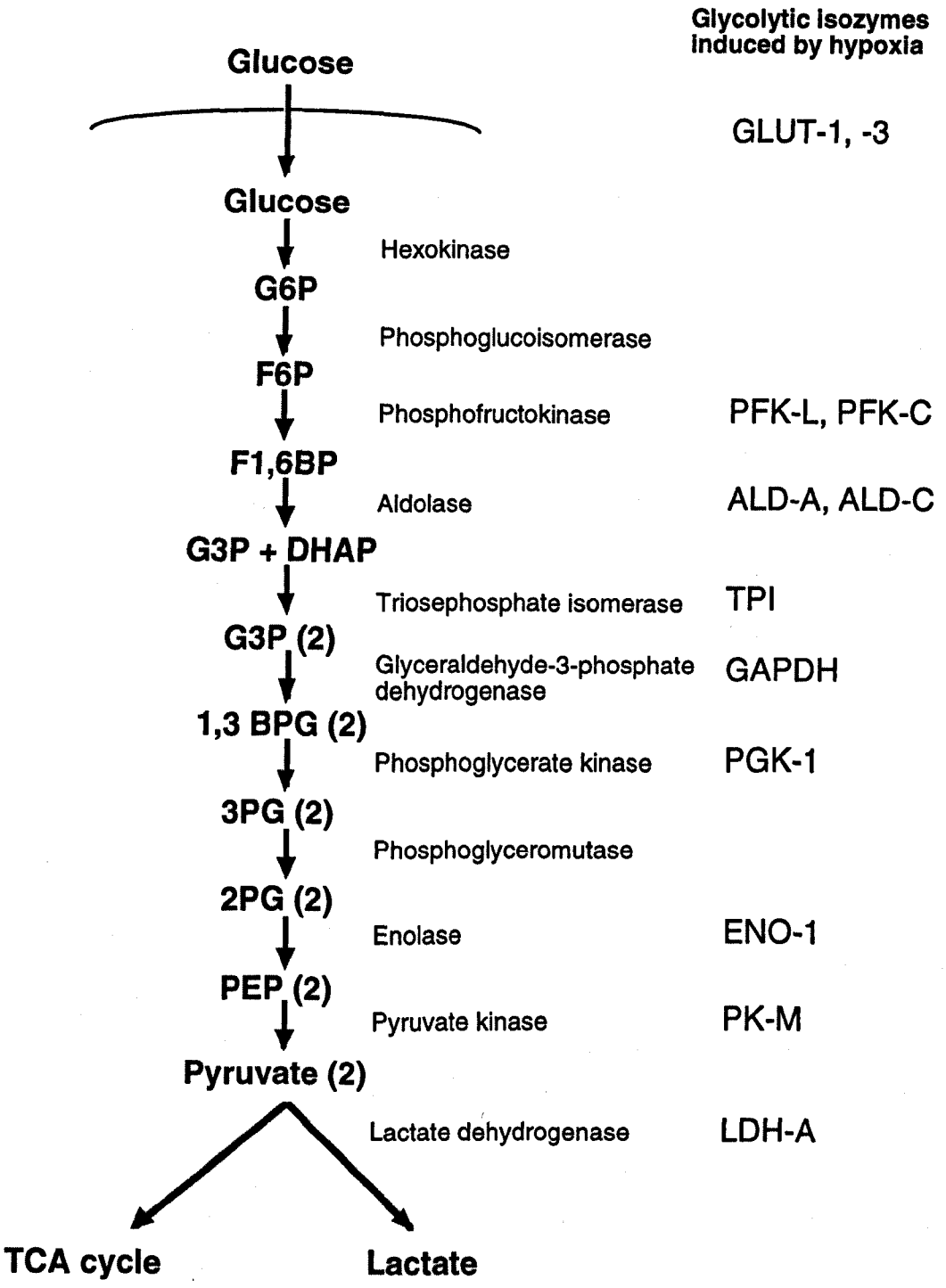


Figure 11: Flow diagram of the isozyme-specific regulation of glycolytic function in response to cellular hypoxia.

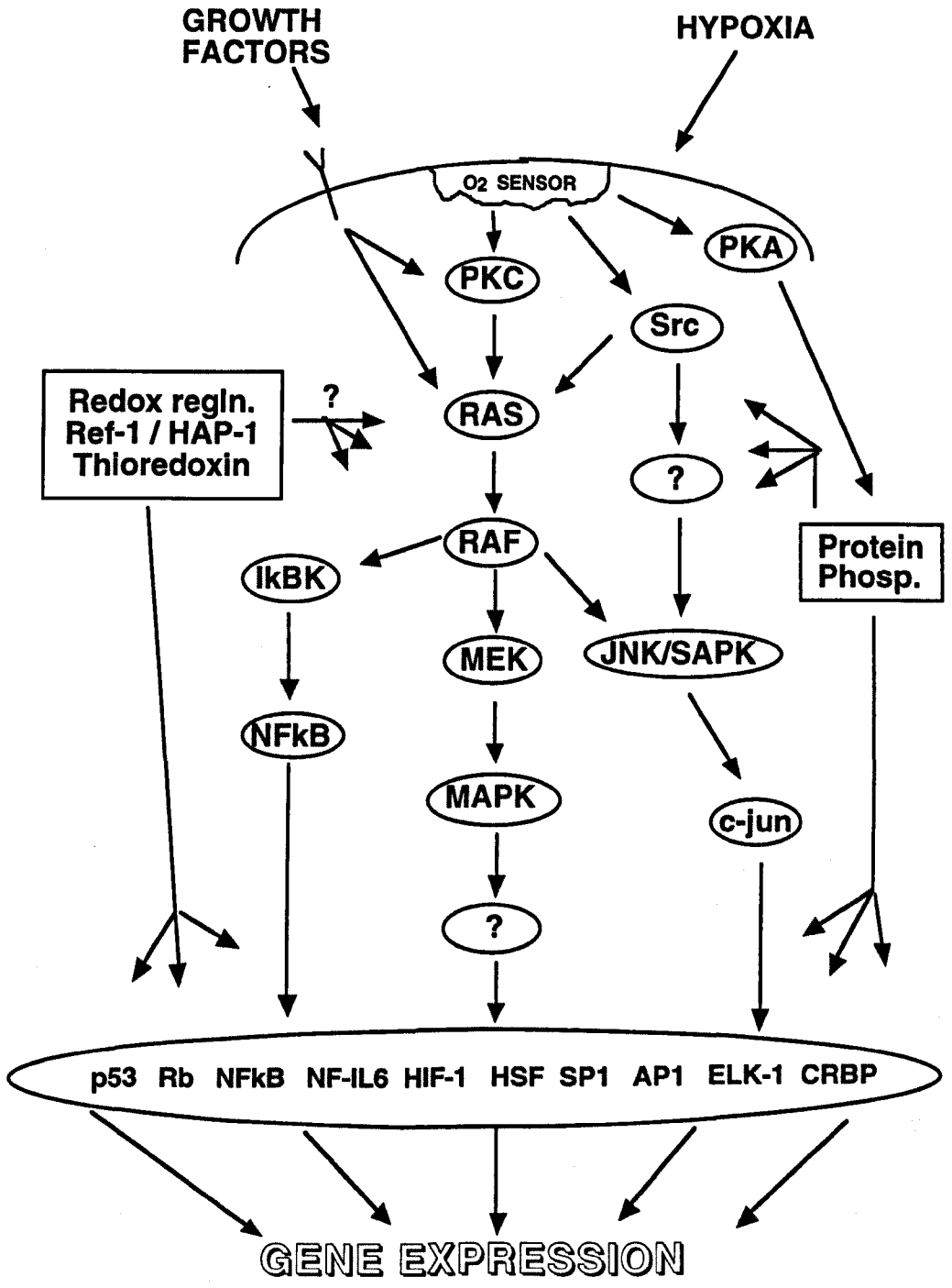
Yet a striking feature of cancer cells is their ability to generate large amounts of lactic acid through enhanced glycolysis, despite the presence of sufficent oxygen (Warberg effect) (Warberg, 1956). Evidence points to a role for certain key oncogenic transcription factors, particularly c-Myc, as well as activated oncogene tyrosine kinases (ras, src, bcr-abl) in the constitutive upregulation of glycolytic flux (Dang *et al.*, 1997). This increase in aerobic glycolysis is not necessarily a reflection of an elevated demand for metabolic energy via glucose catabolism, but is perhaps anabolic, in that glycolytic enzymes themselves or by-products of glycolysis, perform multiple functions that help promote and sustain unregulated proliferation. This is suggested by several observations; in cells transformed by Rous sarcoma virus, ENO-1, PGM and LDH-A are found to be tyrosine phosphorylated (Cooper *et al.*, 1983). Enolase has apparent plasmin-binding functions, while PGM can function as a neuropeptide growth factor. Also, in its phosphorylated form LDH-A localises to the cell nucleus, and has been found to be a single-stranded DNA binding protein with DNA helix-destabilising activity (Zhong and Howard, 1990). Further, pyruvate kinase has beeen shown to be identical to the cell division cycle protein, cdc 19, implying a direct link between glycolysis and cell cycle control (Aon *et al.*, 1995).

1.6.1 Studies of cis-acting elements

The DNA regulatory elements controlling the expression of oxygen-responsive genes have been implicated or defined in most cases, and involve the specific binding and transactivation by various inducible, phosphorylation-dependent and/or redox sensitive transcription factors including; Hypoxia Inducible Factor-1 α/β (HIF-1) (Wang and Semenza, 1993a; 1993b), fos/jun Activator Protein-1 (AP-1) (Goldberg and Schneider, 1994; Yao *et al.*, 1994a; 1994b; Norris and Millhorn, 1992), Nuclear Factor κβ (NF-κβ) (Koong *et al.*, 1994a; 1994b; Yao and O'Dwyer, 1995; Lavrovsky *et al.*, 1994), p53 (Graeber *et al.*, 1994), the CCAAT-enhancer binding protein β (C/EBP-β) from the NF-IL6 family (Ohkawa *et al.*, 1994; Estes *et al.*, 1995), the Heat Shock Transcription Factor (HSTF) (Benjamin *et al.*, 1990; Giaccia *et al.*, 1992) and perhaps the ubiquitous SP-1 transcription factor (Kobayashi *et al.*, 1996; Schafer *et al.*, 1996). Published evidence indicates that only HIF-1 is specifically oxygen-responsive, while the other transcriptional systems appear to contribute to the response to hypoxia *via* its related redox and metabolic changes. Indeed it is not uncommon to find multiple regulatory elements within the same promoter, not all of which are necessarily functional. Evidence also exists for cooperative interactions between some of these transcription factors,

suggesting that the functional induction of gene expression relies, at least in part, on the assembly of a critical concentration of *trans*-acting factors.

Figure 12: Simplified representation of the complex integration of multiple *trans*-acting factors in the response of mammalian cellular systems to changes in oxygen concentration.



1.6.1.1 Hypoxia Inducible Factor-1 (HIF-1)

Hypoxia-inducible factor-1 is a heterodimer (HIF-1α/β) that has been shown to transcriptionally regulate oxygen-responsive genes that contain hypoxia response elements (HREs) (Reviews: Dachs and Stratford, 1996; Bunn and Poyton, 1996; O'Rourke *et al.*, 1997). Oxygen-dependent control of HIF-1 is predominantly achieved by changes in the proteolytic stability and *trans*-activation capacity of the HIF-1α subunit, while HIF-1β is constitutively expressed (O'Rourke *et al.*, 1997). As implied the HIF-1α subunit is regulated at the post-mRNA level, being continuously synthesised and degraded under normoxic conditions (Wenger *et al.*, 1997; Salceda and Caro, 1997). Degradation is mediated, at least in part, by the ubiquitin-proteasome system that is regulated through a defined regulatory domain (Pugh *et al.*, 1997). Stabilisation is dependent upon redox-induced changes (Wang *et al.*, 1995; Huang *et al.*, 1996; Pugh *et al.*, 1997) which is complemented and amplified by post-translational changes in transactivation capacity probably involving other redox and protein phosphorylation events (Wang *et al.*, 1995c; Salceda *et al.*, 1997; Pugh *et al.*, 1997; Jiang *et al.*, 1997). Ultimately, transcriptional activation will depend upon an interrelated series of events which includes nuclear accumulation, dimerisation, DNA binding, co-factor recruitment and transactivation.

The oxygen sensing mechanism is thought to involve a heme-sensor protein (Goldberg *et al.*, 1988; Ehleben *et al.*, 1997), which perhaps functions downstream from a flavoprotein oxidoreductase (Gleadle *et al.*, 1995a). This sensor appears to function universally in all malignant cells studied to date (Maxwell *et al.*, 1993; Firth *et al.*, 1994; Ratcliffe *et al.*, 1997). Functional HIF-1 has been shown to be essential for tumour neovascularisation and growth *in vivo* (Maxwell *et al.*, 1997). HREs are found within a range of genes including growth factors and glycolytic enzymes (Maxwell *et al.*, 1993; Firth *et al.*, 1994; Semenza *et al.*, 1994; Gleadle *et al.*, 1995b; Woods *et al.*, 1997), and has been shown to specifically regulate the transcription of HIF-1 regulated genes in response to hypoxia. Recently several closely related transcription factors to HIF-1α have been described. A molecule with 48% homology, first termed endothelial PAS protein-1 (EPAS-1) has been shown to be induced by hypoxia, and can dimerise with ARNT and activate transcription from similar DNA recognition sequences (Tian *et al.*, 1997). EPAS-1 was also independently identified and reported as HIF-related factor (HRF), being found in murine brain capillary endothelial cells, and bronchial epithelium (Flamme *et al.*, 1997). A further identification of EPAS-1, termed HIF-1α-like factor (HLF) has also been observed in mouse lung, heart and hepatic tissues, although constitutive expression was greater than seen for HIF-1α itself (Ema *et al.*, 1997).

A trimer of the minimal HRE from the mouse phosphogylcerate kinase-1 promoter (Firth *et al.*, 1994) has been utilised in a heterologous promoter context to demonstrate the potential for regulating both marker and therapeutic genes in response to low oxygen conditions (< 2% O_2) (Chapter 5; Dachs *et al.*, 1997). *In vitro*, transcriptional response was shown to be time and oxygen concentration dependent, and no HRE-dependent activity was seen at normal tissue pO_2 (5% O_2). *In vivo*, marker gene expression was restricted to tumour tissues adjacent to areas of necrosis, and single cell electrophoresis of irradiated xenografts demonstrated that only hypoxic tumour cells expressed the marker gene.

1.6.1.2 Activator Protein-1 (AP-1)

A number of oxygen-responsive genes (eg. VEGF, TH, Glut-1, DT-diaphorase, γ-glutamylcysteine synthetase) contain AP1 or AP1-like elements which are binding sites for members of the Fos family (c-Fos, Fos-B, Fra-1, Fra-2) and Jun family (c-Jun, Jun-B, Jun-D) of transcription factors (Herschman 1991; Nakabeppu and Nathans 1991). All the Jun family are capable of binding to AP1 sites as hetero- or homo-dimers, while Fos members can only bind in association with a Jun family member. Fos and Jun can also form selective cross-family heterodimers with ATF/CREB family members (Hai and Curran, 1991).

Hypoxia induces the expression of a number of phase II "detoxifying" enzymes which are coordinately regulated via conserved antioxidant response elements (AREs) (Jaiswal, 1994). These cis-acting regulatory sequences contain two or more copies of the AP1 or AP1-like elements in a short stretch (40-45 bp) of DNA, and have been reported to confer the coordinated induction of mRNAs for both the obligate two-electron reductase DT-diaphorase, and the rate limiting enzyme in glutathione synthesis, γ-glutamylcysteine synthetase (>10-fold @ 24h post) in response to hypoxia (O'Dwyer *et al.*, 1994). The elevations in gene expression were thought to be associated with the hypoxic induction of both c-jun and junD by ~30-fold (max. @ 8h) and c-fos by ~17-fold (max. @ 24h post) in the human adenocarcinoma cell line HT-29 (Yao *et al.*, 1994a, 1994b; Yao and O'Dwyer, 1995). However, the time course of induction of these factors was not identical, in that the Jun proteins were strongly and rapidly induced without the requirement for reoxygenation, while Fos protein was induced more slowely and appeared to require reoxygenation for maximal expression. Furthermore, although association of fos, jun- and jun-D with the DT-diaphorase ARE was demonstrated by EMSA and supershift assays and this binding could be outcompeted by an AP-1 (but not a mutant) oligonucleotide, the AP-1

binding did not follow the time course of DT-diaphorase induction. Several other genes that contain AREs have also been shown to bind c-fos, junD and junB in supershift assays (Li and Jaiswal, 1992a; 1992b; Favreau and Pickett, 1993; Friling *et al.*, 1993), including both rat and murine glutathione-S-transferase Ya and π genes (Li and Jaiswal, 1992b; Friling *et al.*, 1992; Diccianni *et al.*, 1992; Nguyen and Pickett, 1992; Bergelson *et al.*, 1994).

Chronic hypoxia (< 0.01% O_2) has been shown to induce enhanced DNA-binding activity of an AP-1 transcriptional complex (perhaps ATF-2/c-Jun) in SiHa cells, which was correlated with a large depletion (80%) in total intracellular glutathione (Laderoute *et al.*, 1996). The response potentially involved an inducible ATF-2 kinase, and earlier work had identified substantial inductions of the c-jun proto-oncogene in SiHa cells (Ausserer *et al.*, 1994), although these studies failed to demonstrate changes in nuclear protein binding to AP-1 sites of the MT-IIA promoter and a reporter construct containing multimerised AP-1 binding sites was not responsive to chronic hypoxia (12h) (Ausserer *et al.*, 1994). However, AP-1 activation in response to hypoxia has been reported for rat PC-12 cells (Norris and Millhorn, 1995; Mishra *et al.*, 1997) and neonatal rat cardiac myocytes (Webster *et al.*, 1993), although there is suggestion for the obligatory involvement of c-fos (Mishra *et al.*, 1997). Increased transcription of the tyrosine hydroxylase (TH) gene in response to hypoxia is mediated through several regulatory elements in the proximal promoter including an AP-1 and a HIF-1 binding site. A specific c-fos/junB complex binds to this AP-1 element, and mutation of the AP-1 element ablates responsiveness of the TH promoter to hypoxia (Norris and Millhorn, 1995). The available evidence would suggest that the involvement of AP-1 in the hypoxic-responsiveness of genes is not only cell line dependent, but also heterodimer and promoter context-specific.

1.6.1.3 Nuclear factor-κβ (NF-κβ)

NF-κβ complexes are activated by a wide variety of stimuli including cytokines (TNFα, IL-1), lipopolysaccharide, phorbol ester, calcium ionophore, lectins and UV light (Thanos and Maniatis, 1995). Induction of NF-κβ DNA-binding activity appears to occur over a relatively narrow range of oxygen tensions in some cell lines (0.1 - 0.01 % pO_2) (Laderoute *et al.*, 1996). The response to hypoxia does not require *de novo* protein synthesis, but rather, occurs as a consequence of the reversible tyrosine phosphorylation and dissociation of an inhibitory subunit, Iκ-Bα, allowing the NF-κβ dimer to translocate to the nuclear compartment (Koong *et al.*, 1994a). The hypoxia signal is thought to be mediated via a Ras/Raf signal transduction pathway, possibly downstream from c-Src tyrosine kinase, and independent of MAP kinase

(Koong *et al.*, 1994b). The agonists of NF-κβ increase levels of reactive oxygen species (ROS) and deplete cells of reduced glutathione, triggering a common redox signalling pathway. The well documented induction of NF-κβ by oxidants, which stimulates tyrosine phosphorylation of Iκ-B would appear to be in conflict with the limited reports that NF-κβ can be activated by hypoxia.

Nevertheless, NF-κβ activation by hypoxia has been demonstrated in Jurkat leukemia cells, NIH 3T3 fibroblasts, HT-29 adenocarcinoma cells and SiHa squamous carcinoma cells (Koong *et al.*, 1994a; 1994b; Yao and O'Dwyer, 1995; Laderoute *et al.*, 1996). NF-κβ -driven reporter constructs have been shown to respond to $pO_2 < 0.1\%$ over relatively short periods of time (4h) (Koong *et al.*, 1994a; Laderoute *et al.*, 1996). The hypoxic induction of both DT-diaphorase and heme oxygenase-1 may be mediated, in part, by NF-κβ binding (Lavrosky *et al.*, 1994; Yao and O'Dwyer, 1995), although HIF-1 is also involved in the activation of heme oxygenase-1 (Lee *et al.*, 1997a).

1.6.1.4 Other hypoxia-sensitive transcriptional factors

The glucose responsive proteins (GRPs) are endoplasmic reticulum-localised proteins that function as molecular chaperones and calcium binding proteins, responding to a plethora of other stresses including; malfolded proteins, glycosylation block, sulfhydryl reducing agents, oxidative stress, amino acid analogues, viral infection and calcium ionophores (Wooden *et al.*, 1991; Little *et al.*, 1994). These stimuli act through the same cis-acting regulatory elements, implying that common signalling mechanisms are involved in the transcriptional induction of GRPs. The proximal grp78 promoter (-599/+33) has been studied in the context of marker gene expression *in vitro* (Gazit *et al.*, 1995) and shown to be responsive to glucose stavation, hypoxia and acidity (Tannock and Rotin, 1989; Sciandra *et al.*, 1984; Roll *et al.*, 1991; Attenello and Lee, 1984). GRP78 is a major protein induced in solid tumours (Sciandra *et al.*, 1984), and grp78 mRNA levels have been correlated with tumour size (Cai *et al.*, 1993). A large number of CCAAT-like motifs are found in the proximal region of the grp78 promoter and are important for both basal and inducible activity (Little *et al.*, 1994). The nuclear factor CTF/NF-1 can transactivate the promoter through the CCAAT element located closest to the transcription start site. A cyclic-AMP reponsive element (CRE) has also been identified (Alexandra *et al.*, 1991), suggesting that cAMP, phorbol esters, steroids and other cAMP-inducers may be involved in the regulation of the grp78 promoter (Koong *et al.*, 1994d).

Heat shock proteins (HSPs) are also induced by multiple stresses, including; thermal injury, ROIs, sulphydryl reagents, oxidising agents, heavy metals and uncouplers of oxidative phosphorylation. HSPs appear to serve as molecular chaperones, protecting stressed proteins from denaturation. HSP genes are regulated by the redox-sensitive heat-shock transcription factor(s) (Huang *et al.*, 1994), and have been found to be induced by transient hypoxia, although evidence suggests that HSP expression may be more accurately defined as a response to reoxygenation injury (Benjamin *et al.*, 1990; Sciandra *et al.*, 1992).

1.6.1.5 Redox regulation of transcription factors

Hypoxia has been demonstrated to induce expression of the multifunctional redox / DNA repair enzyme HAP1 (also known as Ref-1, APE or APEX) (Walker *et al.*, 1994; Yao *et al.*, 1994b) which possesses both apurinic / apyrimidinic (AP) endonuclease activity and 3' phosphodiesterase activity as well as cellular redox functions (Abate *et al.*, 1990; Xanthoudakis *et al.*, 1992). Consistent with its redox role, HAP1 can substitute for DTT and reductively activate the DNA-binding of oxidised c-Fos and c-Jun (Xanthoudakis *et al.*, 1992). These two properties of HAP1 are located in structurally and functionally distinct domains (Review: Barzilay *et al.*, 1996). It role as a redox regulator of the DNA binding activity of transcription factors can be narrowed to the N-terminal domain from residue 36-62, which is sufficient to reactivate oxidised c-Jun protein (Walker *et al.*, 1993; 1994). Two of the seven cysteine residues in HAP1 (Cys-65 and Cys-95) are also necessary for full activity, and can be targets for thioredoxin (TDX)-mediated reduction (Hirota *et al.*, 1997). TDX is an endogenous redox molecule that can directly associate with HAP1 and consequently reduce it (Quin *et al.*, 1996). Cys-32 and Cys-35 of TDX, which constitute its catalytic centre, are involved in this association (Hirota *et al.*, 1997).

While AP-1 is not a direct substrate of TDX (Xanthoudakis *et al.*, 1992), nuclear translocation of TDX is indispensible for potentiating the AP-1 transcriptional activity, through its association with HAP1 (Hirota *et al.*, 1997). Redox regulation of AP-1 involves a conserved cysteine residue in the DNA binding domain (Abate *et al.*, 1990). Substitution of the cysteine with a serine results in a gain-of-function, which is one of the changes seen in the transforming oncogene v-Jun compared with the non-transforming c-Jun protein (Okuno *et al.*, 1993). This relationship between TDX, HAP1 and AP-1 transcriptional activation suggests a cascade of cellular redox couples that leads to coordinate gene regulation. In support of its wider role, a direct physical interaction between TDX and the DNA-binding loop of NF-κβ p50 subunit has

been demonstrated, suggesting that NF-κβ ia a target for redox regulation by TDX (Qin *et al.*, 1995). Additionally, intracellular expression of both TDX and HAP1 can markedly potentiate the HIF-1 -dependent hypoxic induction of a reporter gene in a cooperative fashion (Huang *et al.*, 1996). In agreement, oxidation or alkylation of the cystine residues in HIF-1 completely eliminated its ability to bind a HRE probe (Wang *et al.*, 1995c). Together this suggests a central role for HAP1, TDX, and by implication TDX reductase, in the regulation of multiple transcription factors via a redox-sensitive signal cascade.

1.6.1.6 Signal transduction - protein phosphorylation

Protein phosphorylation is a widely used mechanism of cellular signal transduction, involving the interplay of a broad repertoire of kinases and phosphatases. Tyrosine phosphorylation, involving c-Src, Ras, Raf and the MAP kinase pathway has been implicated in the hypoxic activation of DNA-binding activity (Koong *et al.*, 1994a; 1994b). Utilising a range of inhibitors, both serine/threonine and tyrosine phosphorylation has been shown to contribute to HIF-1 induction under hypoxic conditions. Genistein, which has an inhibitory effect on many protein kinases including the non-receptor tyrosine kinase c-Src, completely blocks the hypoxic induction of HIF-1 DNA-binding activity (Wang *et al.*, 1995d). V-Src expression has been linked to HIF-1 activation (Jiang *et al.*, 1997a). A selective MEK inhibitor that can prevent phosphorylation of ERK, the terminal kinases of the MAP kinase cascade, also suppressess the hypoxia-induced trans-activation function (but not DNA-binding) of HIF-1 (Salceda *et al.*, 1997). Together these observations suggest an important role for protein phosphorylation in cellular signalling in response to oxygen deprivation.

1.6.1.7 Regulation of messenger RNA stability

mRNA stability is an important site at which gene expression is regulated. Elements critical for stability have been found throughout the mRNA molecule, from the 5' cap structure, which is resistant to RNases, to the 3' poly(A)tail, which must be deadenylated before the mRNA can be degraded. Message degredation is closely associated with translation, such that a minimal level of translation is necessary for mRNA degredation. Thus sequences in the 5' UTR that inhibit translation also inhibit mRNA decay, while sequences in the open reading frame can influence co-translational degredation. The AUUUA-destabilising sequences in the 3' UTR of many unstable mRNAs are the best characterised elements (Sachs, 1993). Evidence suggests that

multiple AUUUA and polypyrimidine sequence motifs in the VEGF mRNA 3' UTR (also present in Epo, GM-CSF, TNFα, HIF-1α, GLUT-1 and tyrosine hydroxylase mRNAs) confer increased stability under hypoxic conditions (Glodberg *et al.*, 1991; Stein *et al.*, 1995; Ikeda *et al.*, 1995; Wang *et al.*, 1997; Levy *et al.*, 1995; Damert *et al.*, 1997; Shaw and Kamen, 1986; Czyzyk-Krzeska *et al.*, 1994). The mechanisms governing mRNA stabilisation are poorly defined but may involve the induction of various molecular chaperones during and following hypoxic stress (Giaccia *et al.*, 1992). A region of the VEGF 3'-UTR forms a hypoxia-inducible RNA-protein complex, which is believed to participate in the post-transcriptional mechanism of mRNA stabilisation (Levy *et al.*, 1997). Negative regulation of mRNA stability has been found to involve the von Hippel-Lindau protein (Gnarra *et al.*, 1996; Iliopoulos *et al.*, 1996).

1.7 Hypoxia and its therapeutic consequences

Multiple factors are involved in the relative resistance of many malignancies to radiotherapy and chemotherapy, including an intrinsic genetically determined resistance and other physiological extrinsic factors, primarily created by inadequate and heterogeneous vascular networks. Thus properties such as blood flow, tissue oxygenation, nutrient supply, pH distribution and bioenergetic status, factors which are usually interrelated, can markedly influence therapeutic response. The presence of regions of low oxygen tension in a variety of human solid tumours is now well established (Dishe, 1985; Gatenby *et al.*, 1988; Vaupel *et al.*, 1991; Nordsmark *et al.*, 1994) and this hypoxia can predispose to failure of treatments with radiotherapy and probably many chemotherapeutic drugs (Hall, 1994; Gatenby *et al.*, 1988; Overgaard, 1992; Hockel *et al.*, 1993a; 1993b; 1996; Okunieff *et al.*, 1993; 1996; Moulder and Rockwell, 1987; Wilson, 1992; Teicher, 1994).

1.7.1 Cytokinetic and apoptotic effects of hypoxia.

Under severe hypoxia no cells enter S-phase and most cells within S-phase remain stationary except in late S-phase, where they are able to complete DNA synthesis (Åmellem and Petterson, 1991). The progression from G_2 through to G_1 is slowed, but cell division is successful irrespective of oxygenation status (Petterson *et al.*, 1986; Åmellem and Petterson, 1993). The transit time from the completion of mitosis to late G_1 is also unaffected by pO_2 status, and cells eventually accumulate at an oxygen-sensitive restriction point at the G_1/S border (Åmellem and Petterson, 1993). Although hypoxia perturbs the energy status of a cell, it appears that lack of energy is not directly responsible for the G_1 arrest observed under such conditions (Löffler,

1985). Cells in S-phase are the most sensitive to a hypoxic insult, and the G_1 arrest under hypoxia plays a fundamental role in protecting cells from its lethal effects (Spiro *et al.*, 1984; Åmellem and Petterson, 1991). This was illustrated in studies where hypoxic cells were stimulated to proliferate by treatment with mitogen stimulating factors, such as phorbol esters (Koong *et al.*, 1994c). Phorbol esters that activated the family of mitogen activated protein kinases (MAP kinases) or extracellular signal regulated protein kinases (ERKs) increased cell proliferation and consequently rendered cells highly sensitive to prolonged chronic hypoxia.

At least two pathways appear to participate in hypoxia-induced cell-cycle arrest; the pyrimidine *de novo* synthesis pathway (Löffler, 1987; Åmellem *et al.*, 1994) and the phosphorylation status of the Retinoblastoma gene product, pRB (Åmellem *et al.*, 1996). Cell-cycle inhibition due to the depletion of intracellular pyrimi dine precursor pools, dCTP and dTTP, (Löffler *et al.*, 1983) may occur through inhibition of the respiratory chain-dependent UMP synthesis at the stage of dihydroorotate dehydrogenase, an enzyme dependent on molecular oxygen for its activation. Ribonucleotide reductase activity is also be influenced by low oxygen tension, but this appears not to be limiting even at $pO_2 < 0.01\%$ (Probst *et al.*, 1989; Åmellem *et al.*, 1994). Under pO_2 ranging from 0.13 to 0.01%, salvagable deoxycytidine or uridine can significantly counteract cell-cycle arrest, almost completely abolishing the hypoxia-induced inhibition of DNA synthesis. The dCTP pool is partially restored (75%) by the addition of deoxycytidine under hypoxic conditions (Löffler *et al.*, 1983). Under severe hypoxia (< 4 ppm O_2) deoxycytidine has no effect on the arrest maintained in G_1 or on the inhibition of cell proliferation in S-phase, but it completely abolishes the delay in initiation of DNA synthesis following reoxygenation (Åmellem *et al.*, 1994). This effect is not seen when deoxycytidine is only present upon reoxygenation suggesting that the dCTP pool is an important G_2-phase signal in the preparation for DNA synthesis upon reoxygenation. The dCTP pool has been implicated in the regulation of the rate of DNA synthesis (Bjursell and Reichard, 1973), and other results also point to the size of the dCTP pool rather than that of the purine deoxynucleotide pools as critical in the regulation of cell proliferation under hypoxia (Probst *et al.*, 1989; Löffler, 1992).

The role of pRB is less clear, since hypophosphorylation and nuclear binding of pRB initiates ~ 4 h after the onset of hypoxia, whereas progression through S-phase is blocked immediately (Åmellem *et al.*, 1996). While the immediate arrest appears to be a consequence of blocking *de novo* synthesis of pyrimidine deoxynucleotides, reentry of G_1-arrested cells into S-phase after reoxygenation, only takes place after pRB deactivation by hyperphosphorylation. It has been proposed that pRB phosphorylation status serves as a stress indicator and can take over as the main negative regulator of cell-cycle progression during prolonged hypoxia. This

hypoxia-induced dephosphorylation of pRB appears to be independent of p53, and may be due to activation of a specific pRB-phosphatase, although $p21^{WAF1/CIP1}$ or other cdk inhibitors can not be excluded from contributing to this p53-independent growth arrest mechanism (Åmellem *et al.*, 1996).

Hypoxia can also induce the hyperphosphorylation of the p34 subunit of replication protein A (RPA), a single-stranded DNA binding protein that plays a central role in DNA replication. RPA hyperphosphorylation inhibits replicon initiation and may be the signal for early S-phase arrest under hypoxic conditions (Giaccia, 1996). Alternatively the hypoxia-inducible protein GADD45, (Price and Calderwood, 1992), is known to bind proliferating cell nuclear antigen (PCNA) and inhibit PCNA's association with replication factor C, an association necessary for recruitment of DNA polymerase δ (Tsurimoto *et al.*, 1990). This would in principle prevent the complete assembly of the initiation complex necessary for DNA elongation, thereby inhibiting DNA synthesis (Graeber *et al.*, 1996; Giaccia, 1996).

While cell-cycle arrest is independent of the activity of p53 tumour suppressor gene, hypoxia stimulates the nuclear accumulation of p53 protein, increased p53 DNA binding and transactivation function in cells that do not possess mutant p53 (p53mt) (Graeber *et al.*, 1994; Giaccia, 1996). This has recently been demonstrated to involve the direct interaction between p53wt and HIF-1, resulting in p53wt protein stabilisation (An *et al.*, 1998). P53, but not p53mt, is also activated by the hypoxia-inducible redox/repair enzyme HAP1, through a noncovalent interaction that involves both redox-dependent and -independent mechanisms (Jayaraman *et al.*, 1997). Hypoxia induces apoptosis in a p53-dependent manner, and thus it can select for individual clonogens that harbour disfunctional p53mt, such that they will eventually become the dominant genotype. This hypoxia-mediated selection of cells with diminished apoptotic potential has important implications for the response of solid tumours to therapy (Graeber *et al.*, 1996; Giaccia, 1996). Graeber *et al.* (1996) demonstrated that apoptosis in wild-type p53 tumours was distal to blood vessels and correlated with binding of the hypoxia-specific marker EF5. In contrast p53mt tumours showed a lower frequency of apoptosis (3.4-fold) in EF5-positive regions, and no relationship was seen with blood vessel density. Consequently hypoxia was acting as a physiological selective agent against apoptosis-competent cells, thereby promoting the expansion of cells defective in apoptosis. Since these same pathways govern the apoptotic response of cells to radiation and chemotherapeutic agents (Lowe *et al.*, 1993; Lotem and Sachs, 1993), hypoxia may drive the acquisitionof apoptosis-incompetent solid tumours, resulting in the poor response of solid tumours to multiple therapies (Lowe *et al.*, 1994).

1.7.2 Radiotherapy.

The importance of tumour oxygenation in radiocurability was first proposed by Gray in 1953, and its role in radiotherapy has since been recognised both in experimental systems (Brown, 1979; Moulder and Rockwell, 1984, 1987; Rockwell and Moulder, 1990; Horsman *et al.*, 1993; Nordsmark *et al.*, 1995) and clinically (Gatenby *et al.*, 1988; Overgaard, 1992; Okunieff *et al.*, 1993; Hockel *et al.*, 1993a; 1993b; 1996; Brizel *et al.*, 1996). Anoxic cells (< 10 ppm. O_2) are approximately 2.5 to 3-fold more radioresistant than normoxic cells; that is, a 2.5 to 3 times larger radiation dose is required to achieve the same reduction in clonogenicity or effective tumour control (Figure 11-12). Half-maximal sensitivity (K value) is seen at about 0.3 % O_2 (3000 p.p.m. O_2, 2.1 mm Hg) (Review: Hall, 1994). This phenomenon, known as the oxygen enhancement ratio (Tubiana *et al.*, 1990), is thought primarily to result from oxygen's ability to react with and chemically modify the initial radiation-induced DNA radical (Zhang *et al.*, 1995). If oxygen is present it reacts with the ionised target molecules generating organic peroxide lesions, which are thought to be significantly less repairable forms of damage. For the "oxygen fixation" effect to be observed, oxygen must be present during, or within a millisecond after, the radiation exposure (Michael *et al.*, 1973).

The recent development of methodologies incorporating the technique of gas chromatography-mass spectrometry (GC-MS) has facilitated the detailed analysis of free radical induced DNA damage (Dizdaroglu, 1985; 1991). This technique permits measurement of a large number of products in the same sample of DNA or chromatin in a single analysis, and allows the chemical characterisation and quantification of base-derived and sugar-derived products as well as DNA-protein cross-links. GC-MS analysis of chromatonised DNA following exposure to ionising irradiation, has demonstrated the formation of various pyrimidine-derived and purine-derived modified bases, the yields and characteristics of which have been shown to be dependent upon factors such as the type of irradiation, DNA conformation and the direct radical environment (Fuciarelli *et al.*, 1990; Gajewski *et al.*, 1989; 1990). The modifications of DNA bases through addition or abstraction by ionising radiation-generated free radicals (eg. $OH^{\bullet}$, e^{-}_{aq}, H atom) is strongly influenced by the presence of an oxic *vs.* an anoxic enviroment. Different radical enviroments can be provided by saturating an aqueous suspension of chromatin with various gases (argon, air, N_2O, N_2O & O_2) and has profound effects on the types of DNA base products formed and their quantitative yields. The presence of oxygen increases the yields of most products with the exception of formamidopyrimidines. Some products (eg. 8-hydroxyguanine) are produced almost exclusively in the presence of oxygen, while others are produced under both conditions. When oxygen is present, peroxyl radicals are formed by

addition of molecular oxygen to sugar or base radicals of DNA. Further short chain reactions of DNA radicals may occur but these will depend upon the types of radicals, their reaction partners and the reaction environment. This results in the formation of a wide variety of final products through a spectrum of different mechanisms. Many of these modified bases have been shown to be produced in γ-irradiated cultured mammalian cells (Dizdaroglu *et al.*, 1987; Nackerdien *et al.*, 1992). DNA-protein cross-links are also formed through radical-radical and radical addition reactions, examples of which, involving thymine and tyrosine, have been identified in the chromatin of γ-irradiated mammalian cells *in vitro* (Olinski *et al.*, 1992).

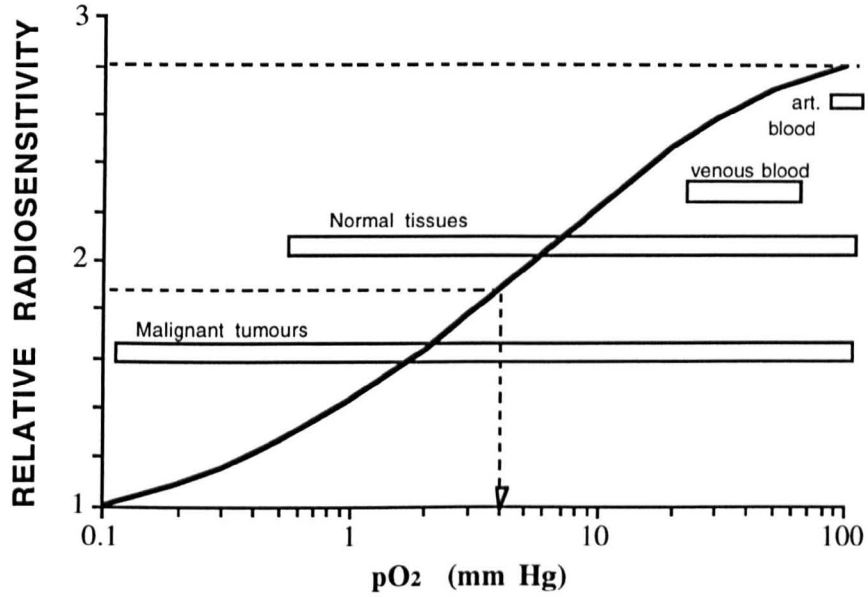


Figure 11. Relative radiosensitivity as a function of oxygen partial pressure (pO2). The range in values usually found in blood, normal tissues, and malignant tissues is illustrated. The pO2 at which the sensitizing effect is half-maximal is indicated. (Adapted from Vaupel, 1989a).

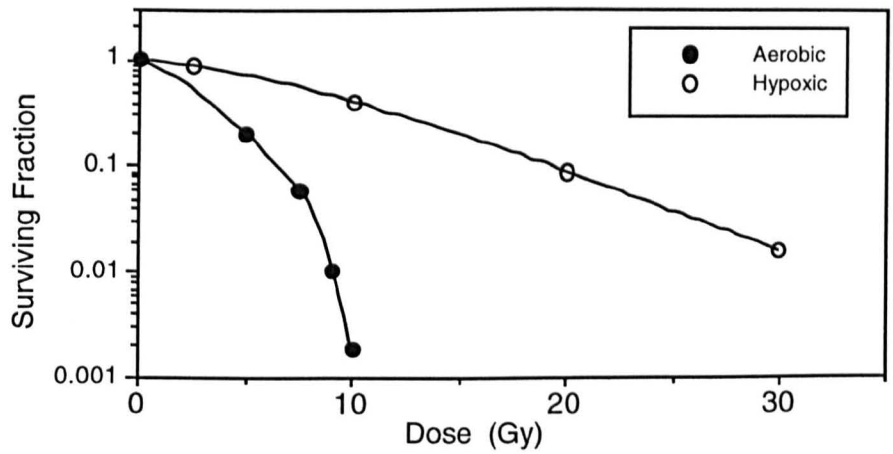


Figure 12. Differential cell kill under aerated or hypoxic ratio, resulting in an oxygen enhancement ratio of 2.5 - 3.5. (Adapted from Hall, 1994).

The large body of experimental evidence defining a role for molecular oxygen in the radioresponse of mammalian cells would strongly suggest that the low oxygen tensions found in many human tumours will alter response to radiation therapy. However the *in vivo* situation is more complex and a number of clinical parameters are known to influence the probability of tumour control, including; tumour size, histological grade, clinical stage, patient performance status and the skill of the physician providing the radiation treatment. Other metabolic and physiological variables, which like pO_2 distribution may be intrinsically heterogeneous, are believed to impact on treatment response. These include glucose delivery and utilisation rates, glutathione content, intracellular pH, and blood flow. Also cell cycle alterations and the rate of clonal repopulation following a radiation dose, along with the potential for intrinsic and acquired radioresistance, will all impact on the final treatment outcome. Thus it has been argued that the intrinsic heterogeneity of both intra- and inter-tumour response will dominate as a cause of failure (Hendry and Thames, 1992), and the impact of hypoxia will effectively be negligible. Considering the wide range in intrinsic radiation sensitivity that is suggested from *in vitro* studies, governed by multiple biological factors such as repair capacity, altered apoptotic potential, modified proliferation and cell cycle effects as well as changes in expression of oncogenes, such a contention seems feasible. Furthermore, it is anticipated that the impact of hypoxia might be decreased in a fractionated radiation regimen, where reoxygenation can occur between doses, and other biological responses between fractions such as redistribution of the cell cycle, repopulation and radiation damage repair might play a dominant role in overall response (Suit *et al.*, 1988). However, despite all these considerations, attempts to use intrinsic radiation sensitivity to predict radiation treatment response have been of limited value (Muller *et al.*, 1985; Peters *et al.*, 1985; 1986; West *et al.*, 1989), and the clinical application of hyper-fractionated regimens, while providing a survival benefit, have not met with the success that optimistic rationalisations might suggest. It is argued that complete reoxygenation between fractions is rare (Finkelstein and Glatstein, 1988), and the marked radiation protection afforded by a hypoxic enviroment, will be a key determinant of many, if not most, treatment failures.

The accumulating clinical evidence of the actual relationship between tumour oxygen status and outcome of treatment overwhelmingly points to tumour oxygenation as an extremely important modifier of the slope of the dose-response curve in some tumour types. Gatenby *et al.* (1988) measured the oxygenation of cervical lymph nodes metastasis in patients with squamous cell carcinoma of the head and neck and showed a significant relationship between low mean intratumoural pO_2 values and failure to respond to fractionated radiotherapy. Furthermore, the proportion of tumour tissue readings with a pO_2 of < 8 mm Hg (1% $pO_2 \equiv$ 7.6 mm Hg) was related to the probability of achieving a clinically complete response after radiotherapy. In

support of these observations, Okunieff *et al.* (1993) showed that low-pO_2 distribution in breast tumours was a significant independent prognosticator, and alone was sufficient to explain the incidence of local recurrence following radiotherapy. Hoeckel *et al.* (1993; 1996) found that patients with advanced-stage cervical carcinoma, who had tumours with a median pO_2 of less than 10 mm Hg had significantly worse overall survival (P= 0.0039) and relapse-free survival (P= 0.0085) than those individuals whose tumours were relatively well oxygenated (> 10 mm Hg). Remarkably, the disadvantage in outcome for low-pO_2 tumours was substantial irrespective of whether the primary treatment was fractionated radiotherapy or radical surgery. For example the 27-month disease-free survival after radical surgery was 82% in "non-hypoxic" tumours *vs.* 27% in those with "hypoxic" tumours (P= 0.01). This suggests that hypoxia may identify tumours with an aggressive phenotype, independent of the therapy received. Indeed, multivariate Cox regression analysis confirmed tumour oxygenation status as the most powerful pretreatment prognostic indicator and demonstrated the strong predictive value of tumour oxygenation measurements on recurrence-free and overall survival. In contrast, pO_2 status was independent of clinical tumour grade (P= 1.0), tumour size (P= 1.0), histological type (P= 0.416) and differentiation (P= 0.296). Neither did pretreatment pO_2 status relate to any patient demographics including; age, menopausal status, or hemoglobin levels. A suggestive observation from the histopathological examination of the subset of surgically resected specimens (n=47), was that low-pO_2 tumours exhibited larger tumour extensions (P= 0.036) and more frequent occult parametrial spread (P= 0.026), as well as greater probability of pronounced lymph-vascular space involvement (P= 0.036) relative to their non-hypoxic counterparts. Although locoregional failure was the dominant source of recurrence in this study, it was noted that in the surgically treated subpopulation, 8 of 13 recurrences of the hypoxic tumours had a metastatic component, as compared to only 2 of 7 recurrences in the non-hypoxic tumours. This is interesting in view of the report by Brizel *et al.* (1996) where pretreatment tumour pO_2 measurements in soft tissue sarcoma predicted the likelihood of distant relapse, but not local failure. The actuarial 18-month disease free survival in the nonhypoxic *vs.* the hypoxic tumours was 70% *vs.* 35% respectively.

1.7.3 Chemotherapy

Less clinical evidence is available regarding the impact of hypoxia in the failure of chemotherapy. However there are compelling reasons (Moulder and Rockwell, 1987; Wilson, 1992; Teicher, 1994; 1995) and considerable experimental suggestion (Smith *et al.*, 1979; Teicher *et al.*, 1981; Tannock and Guttman, 1981; Kennedy, 1987; Sartorelli, 1988; Grau and

Overgaard, 1988; Hockel *et al.*, 1993) to anticipate that hypoxic cells are resistant to some types of cytotoxic drugs, not least because a high proportion of hypoxic cells are non-cycling and many chemotherapeutic agents are preferential mitotic poisons. An exposure period of 16h hypoxia can reduce DNA synthesis by 80-90% *in vitro* (Krtolica and Ludlow, 1996; Graeber *et al.*, 1994), irrespective of cell cycle, providing marked protection from many commonly employed anti-metabolites. This is evidenced by reports showing that exponentially growing cells *in vitro*, when rendered chronically hypoxic during drug exposure, are more resistant to many chemotherapeutic agents, particularly anti-metabolites and DNA-interchelators. These include actinomycin D, doxorubicin, etoposide, mitoxantrone, vincristine, m-AMSA, taxol, 5-fluorouracil, arabinoside, procarbazine, streptonigrin, and bleomycin (Roizin-Towle and Hall, 1978; Smith *et al.*, 1979; Adams *et al.*, 1980; Teicher *et al.*, 1981; Teicher *et al.*, 1985; Shen *et al.*, 1987; Hughes *et al.*, 1989; Herman *et al.*, 1992; Bisset and Kaye, 1993; Teicher, 1994). With redox active agents such as bleomycin, streptonigrin (and perhaps etoposide), oxygen itself is directly involved in the mechanism of toxicity (Teicher *et al.*, 1981; 1985), but for the majority of chemotherapeutics, resistance is not strictly related to the absence of molecular oxygen during drug exposure *per se*, and may involve the induction of multiple early stress response proteins, complex modulations of cell cycle progression and changes in recognition and/or response to DNA damage. This capacity for rapid adaptation also extends to the acquisition of temporary drug resistance as a post-hypoxic characteristic. This transient phenomenon has been reported for doxorubicin, methotrexate, 5-Fluorouracil, actinomycin-D, etoposide, BCNU, and cisplatin, in various murine and human cell lines models (Smith *et al.*, 1980; Wilson *et al.*, 1989; Luk *et al.*, 1990; Sakata *et al.*, 1991; Kalra *et al.*, 1993; Sanna and Rofstad, 1994; Liang, 1996).

The biological mechanisms underlying these changes in drug sensitivity are not well understood but evidence suggests such hypoxia-induced resistance may reflect changes in metabolic and/or repair processes, particularly as a number of stress related proteins are known to be expressed in response to hypoxic stress (Heacock and Sutherland, 1986; Dachs and Stratford, 1996). As discussed earlier, hypoxia induces the expression of a number of detoxifying enzymes, the transcription of which are coordinately regulated via AREs (Jaiswal, 1994), including; γ-glutamylcysteine synthetase, glutathione-S-transferase Ya and π genes (Li and Jaiswal, 1992b; Friling *et al.*, 1992; Diccianni *et al.*, 1992; Nguyen and Pickett, 1992; Bergelson *et al.*, 1994; O'Dwyer *et al.*, 1994). These enzymes are known to be involved in the glutathione-mediated inactivation of many chemotherapeutic agents (Hodgkiss and Middleton, 1985; Wang and Tew, 1985; Miyazaki *et al.*, 1990; Nakagawa *et al.*, 1990; Schecter *et al.*, 1993; Tew, 1994; Chen and Waxman, 1995; Dirven *et al.*, 1995). Metallothionein IIA (MT-

IIA), a stress-inducible protein known to be involved in resistance to cisplatin (Kelley *et al.*, 1988; Satoh *et al.*, 1993), doxorubicin (Naganuma *et al.*, 1988) and mitomycin C (Lohrer and Robson, 1989) is also up-regulated by chronic hypoxia (Murphy *et al.*, 1994). A proximal fragment of the human MT-IIA promoter (0.2 kb) was demonstrated to confer hypoxia responsiveness to a reporter gene in A431 human squamous carcinoma cells (Murphy *et al.*, 1994). These responses may involve the sensing of perturbations in cellular redox state, that ultimately result in the phosphorylation of specific AP-1 transcriptional complexes (Bergelson *et al.*, 1994; Laderoute *et al.*, 1996). Other known targets of Fos/Jun action include dTMP synthase and topoisomerase I which are implicated in resistance to 5-Fluorouracil and camptothecin respectively. The fact that both the GST gene and the MDR gene contain AP-1 binding sites, perhaps suggest that Fos/Jun may contribute towards a coordinated acquisition of drug resistance (Dietel, 1993).

Hypoxia has also been demonstrated to induce expression of the cellular redox and AP endonuclease repair enzyme, HAP1 (section 1.6.1.5) (Walker *et al.*, 1994; Yao *et al.*, 1994b; Abate *et al.*, 1990; Xanthoudakis *et al.*, 1992). Depletion of HAP1 protein via expression of antisense HAP1 RNA renders HeLa cells hypersensitive to killing by a wide range of cytotoxic agents including simple alkylating agents, peroxides, bleomycin, the redox cycling drug menadione and X-rays (Walker *et al.*, 1994; Chen and Olkowski, 1994). HAP1 has an important functional role *in vivo* modification of the apurinic / apyrimidinic (AP) sites generated by the action of DNA glycosylases in the DNA base excision repair pathway (Review: Barzilay and Hickson, 1995). DNA glycosylases recognise and remove a wide range of small DNA adducts (eg. alkylation) which are generally associated with a single base residue, as well as oxidative damage involving base modification and/or disruption of the sugarphosphate backbone (Review: Demple and Harrison, 1994). The AP-sites resulting from this base excision prompt the 5' hydrolytic cleavage of the sugar-phosphate backbone by HAP1, generating both a 3' OH site for subsequent priming of a DNA repair polymerase and also a 5' deoxyribose phosphate group, which then requires further modification by a deoxyribose phosphodiesterase. Of note, DNA polymerase β which is thought to perform most of the DNA synthesis associated with base excision repair also possesses efficient deoxyribose phosphodiesterase activity (Matsumoto and Kim, 1995).

Interestingly, the HAP1-depleted HeLa cells not only demonstrate sensitivity to hyperoxia (95% O_2) but are also hypersensitive to hypoxic exposure (1% O_2). The functional involvement of HAP1 in protection from hypoxia-induced cell death is less obviously explained. It is possible that hypoxia produces as yet unidentified DNA lesion(s) that require the action of

HAP1 as a DNA repair enzyme (Russo *et al.*, 1995). More likely, its partially defined redox regulatory role in transcriptional activation (see section 1.6) may be an essential component for the coordinated response of a large number of other oxygen responsive proteins that serve to protect cells under redox-stress. As a whole, experimental observations point to the cellular redox functions of HAP1 as being critical in the coordinate redox-regulation of several oxygen-responsive transcription factor families, including; fos/jun, NF-κβ, and HIF-1 (Abate *et al.*, 1990; Xanthoudakis and Curran, 1992; Hirota *et al.*, 1997; Xanthoudakis *et al.*, 1992; Huang *et al.*, 1996) These *trans*-acting factors represent a major collection of redox-sensitive nuclear proteins that govern the transcriptional response of a plethora of genes to oxygen deprivation, some of which are known to contribute to multidrug resistance.

Other *in vivo* pharmacokinetic considerations may also contribute to the protection of hypoxic cells during systemically delivered chemotherapy. Hypoxic tumour cells are more likely to be distal to functionalvasculatureat the time of drug administration and gradients of drug penetration can arise (Kennedy, 1987). It has been demonstrated that cells distal from functional blood vessels (as determined by the limit of Hoechst 33342 penetration) are spared by many antitumour agents *in vivo* (Teicher *et al.*, 1990). Since many chemotherapy agents have a narrow therapeutic index, even modest heterogeneity in tumour drug exposure may have important implications for clinical drug resistance. Further, the possible relationship between interstitial fluid pressure (Jain, 1987) and reduced perfusion rates and tissue pO_2 (Lee *et al.*, 1992; Roh *et al.*, 1991a) suggests that hypoxic tumours are more likely to resist the influx of systemically administered agents.

1.8 Hypoxia-induced genetic instability and tumour progression.

Experimental and clinical evidence points to hypoxia as a critical component, not only in the resistance of solid tumours to conventional therapies, but as a potent physiological stimuli that promotes tumour progression, both driving genetic diversity and selecting for aggressive neoplastic phenotypes (Hill, 1990; Graeber *et al.*, 1994; Hockel *et al.*, 1996). Hypoxia appears not only to provide an environmentdirectly facilitating drug resistance as well as temporarily inducing a spectrum of drug resistance-associated genes, but can also encourage the evolution of stable phenotypic change characteristic of permanent chemotherapeutic drug resistance. Anoxia has been shown to enhance DNA strand breaks, leading to increased genomic instability (Russo *et al.*, 1995). A marked enhancement in the frequency of methotrexate resistance and dihydrofolate reductase (DHFR) gene amplification has been observed *in vitro*, with acquisition

of resistance being related to the duration of both hypoxia and re-oxygenation (Rice *et al.*, 1986). In further studies, hypoxia was shown to markedly enhance the frequencies of simultaneous resistance to methotrexate and doxorubicin, and was related to both DHFR and p-glycoprotein gene amplification (Rice *et al.*, 1987).

Loss of p53 also appears to enhance certain types of genetic instability and has been linked to enhanced aneuploidy, gene amplification and recombination events, all of which may then contribute to further tumour progression (Review: Hartwell and Kastan, 1994). Loss of functional p53 has been demonstrated to occur following cycles of hypoxia and reoxygenation *in vitro* (Graeber *et al.*, 1996). In addition, the mutation of p53 during tumour progression results in the elimination of certain signals which usually promote apoptotic cell death and consequently some tumours can become inherently resistant to chemotherapy or radiation therapy-induced apoptosis.

Cell cycle perturbations and DNA overreplication arise as a consequence of hypoxia, and have been related to enhanced metastatic potential of fibrosarcoma and melanoma cells (Young *et al.*, 1988). Metastatic potential may also be promoted through the hypoxia-induced hyperploidy (Rofstad *et al.*, 1996) and other genotypic changes, phenomena which are also associated with the evolution of acquired multidrug resistance. Hypoxia might also potentiate the capacity for metastatic spread from local disease sites, through the induction of vascular permeabilising factors such as VEGF or proteolytic enzymes, including the cathepsins (Liotta and Stetler-Stevenson, 1991; Mareel *et al.*, 1993). Indeed, transient hypoxia increased the total cathepsin B + L levels in murine KHT cells, which was correlated with invasive capacity (Cuvier *et al.*, 1997). Glucose starvation and acidosis have also been found to enhance the metastatic potential of KHT cells *in vivo* (Schappack *et al.*, 1991). The relationship between proteolysis and invasion has been firmly established for many tumour types (Mignatti and Rifkin, 1993). Clinical evidence in support of a role for hypoxia in promoting metastatic spread is now just beginning to emerge. It was recently reported that the pretreatment oxygenation status of soft tissue sarcomas predicted the probability of distant relapse, but not local failure (Brizel *et al.*, 1996). The probability of distant metastasis was twice as high for tumours with median pO_2 of < 10 mm Hg relative to those with median pO_2 > 10 mm Hg. The average median pO_2 of 7.5 mm Hg for those patients that relapsed was significantly lower than the average median of 20 mm Hg for those that did not (P= 0.03). All treatment failures were distant lung metastases, with only one patient subsequently developing local recurrence. Since all the tumours studied were high grade it was suggested that hypoxia may be a valid independent prognostic indicator. In agreement Höckel *et al.* (1996) observed that low-pO_2 cervical tumours exhibited larger tumour

extensions and more frequent parametrial spread and lymph-vascular space involvement, compared to well-oxygenated tumours of similar clinical grade and size. Interestingly, high lactate levels in cervical cancer have also been correlated with the incidence of metastatic disease (Schwickert *et al.*, 1995).

1.9 Reversing the clinical radioresistance of solid tumours

Methods designed to overcome hypoxia-dependent radioresistance have included the use of hyperbaric oxygen, the development of oxygen mimetic radiosensitizers (Adams, 1976; Stratford, 1992) and more recently, the combination of nicotinamide (vitamin B derivative) and carbogen (95% O_2 and 5% CO_2) in an attempt to improve tumour oxygenation during radiotherapy (Chaplin *et al.*, 1991).

A meta-analysis of 50 clinical trials using nitroimidazoles as hypoxic cell radiosensitisers has demonstrated a modest survival benefit in head and neck and bladder, but not in cervical, lung, oesophagal or CNS tumours (Overgaard, 1994). For all disease sites there was an overall survival benefit of 2.8 % (P = 0.02). This was closely correlated to the probability of local control, where hypoxic cell sensitisers provided an overall improvement of 3.9 % (P = 0.005), giving benefit in head and neck and bladder tumours, but not in cervix, lung or oesophagus. Meta-analysis of 17 randomised trials comparing hyperbaric oxygen with conventional radiotherapy revealed a similar influence of tumour oxygenation on the probability of local control (Overgaard, 1995). Overall a 6.6 % (P = 0.003) improvement in local disease control was seen.

Putting all the radiosensitisation trials together, there is a clear improvement in both local tumour control and survival of 4.6 % (P = 0.00001) and 2.8 % (P= 0.005), respectively (Review: Saunders and Dishe, 1996). While this is a modest gain in treatment outcome, it nonetheless confirms that hypoxia is a contributory factor in the failure of radiotherapy. It must be considered that patient selection was not carried out for these trials, such that a proportion of the treated tumours would not have harboured a therapeutically relevent concentration of radioresistant hypoxic cells, and thus recieved no benefit from the improvement in tumour oxygenation or the application of oxygen mimetics.

An appreciation of the contributions of both chronic (diffusion-limited) and acute (perfusion-limited) hypoxia in solid tumours has lead to the application of combinatorial

treatments to cooperatively overcome both mechanisms of radioresistance. Nicotinamide is thought to maintain vascular tone, and thus reduces the incidence of temporary vascular shutdown (Horsman *et al.*, 1989). The plugging of microvessels by relatively rigid leucocytes which may also contribute to the proportion of non-perfused vessels, is also partially overcome by nicotinamide (Honess *et al.*, 1996). In animal studies, nicotinamide enhanced local tumour control with radiotherapy (enhancement ratio ~1.2), and was particularly effective when carbogen breathing was included in the treatment (enhancement ratio ~1.6 - 1.8) (Kjellen *et al.*, 1991). The combination of nicotinamide and carbogen has recently been demonstrated to improve tumour perfusion in human subjects, relative to each treatment alone, or no treatment (Powell *et al.*, 1997). Accelerated radiotherapy, when utilised in combination with carbogen and nicotinamide (ARCON), has demonstrated an enhancement ratio of 2.8 in animal tumour models (Rojas *et al.*, 1993), which is better than any other achieved using multi-fraction regimes in similar models (Rojas, 1992). ARCON is currently in European wide clinical trials (Saunders and Dishe, 1996).

1.10 Bioreductive drugs as hypoxia selective cytotoxins.

Since severe hypoxia is largely restricted to solid tumours, its exploitation is an attractive basis for therapeutic selectivity in cancer treatment. As an alternative strategy to using radiosensitisers or improving tumour oxygenation, strategies which by their nature will only restore cellular radiosensitivity to that of the oxygenated population, has been to develop agents that exhibit selective toxicity towards hypoxic cells (Adams and Stratford, 1986; 1994; Stratford *et al.*, 1986; Brown and Siim, 1996; Denny *et al.*, 1996) through the exploitation of enzyme-mediated reductive activation of drugs at low oxygen tensions (Kennedy, 1987; Workman, 1992; Workman and Stratford, 1993).

Five types of metabolic biotransformation are currently known to be inhibited by molecular oxygen, and form the basis for the design of hypoxia-selective cytotoxins (HSCs) (Review: Denny *et al.*, 1996). This includes reduction of nitro(hetero)aromatics, aliphatic and aromatic N-oxides, quinones and transition metal complexes. The hypoxic selectivity of these agents relies upon the inactivation of a one-electron reduction intermediate through the reoxidation by molecular oxygen. Thus the "futile cycling" that occurs in the presence of oxygen inhibits the net reduction of the parent compound in aerobic cells. Selectivity arises because the reduction products of these prodrugs are significantly more cytotoxic than the reactive oxygen species generated as a consequence of rapid back-oxidation. The tertiary

amine (aliphatic) N-oxides, including AQ4N and nitracine N-oxide are notable exceptions to this mechanism since they require concerted two-electron reduction in order to become activated, but the biotransformation is still sensitive to oxygen (Patterson, 1993; Patterson *et al.*, 1994; Wilson *et al.*, 1996). Quinones can be activated by either one- or two-electron reductions to the semiquinone or hydroquinone respectively. Direct two-electron reduction of this class of compounds is not inhibited by oxygen, which reflects a distinction in the enzymology of aliphatic N-oxide *vs.* quinone metabolism. The contribution of the hydroquinone to cytotoxicity is unclear, and the semiquinone is generally considered the proximal cytotoxin (Pan *et al.*, 1984; Powis, 1989; Butler *et al.*, 1996).

Classes of compound that are in, have or are being considered for entry into clinical trial as bioreductive HSCs include; (I) CI-1010, the R enantiomer of RB6145, the lead compound from a series of dual-function, alkylating 2-nitroimidazoles (Adams *et al.*, 1984; Jenkins *et al.*, 1990; Cole *et al.*, 1990; 1992); (II) quinones such as the mitomycin C analogue porfiromycin (Rockwell *et al.*, 1988) and the indoloquinone EO9 (Hendriks *et al.*, 1993; Smitskamp-Wilms *et al.*, 1996); (III) the lead compound in a series of benzotriazene-di-*N*-oxide analogues, tirapazamine (TPZ, SR4233, WIN 59075) (Brown and Lemmon, 1991; Brown, 1993); and (IV) an aliphatic N-oxide analogue of mitoxantrone, AQ4N (Patterson *et al.*, 1994). For these agents to be effective within hypoxic cells they require metabolic activation, catalysed by the cellular complement of reductase enzymes. These may include isozymes of the cytochrome P450 system, NADPH:cytochrome P450 reductase, NADH:cytochrome B_5 reductase, xanthine oxidase, aldehyde oxidase, glutathione reductase, succinic dehydrogenase, NADH:dehydrogenase, xanthine dehydrogenase and DT-diaphorase (Brown, 1993; Workman, 1992; Walton *et al.*, 1989; Hodnick and Sartorelli, 1993; Gustafson and Pritsos, 1993; Robertson *et al.*, 1994; Bailey *et al.*, 1992; 1994; Patterson *et al.*, 1995b; Fitzsimmons *et al.*, 1996; Patterson *et al.*, 1997).

Hypoxic cells constitute only a minor proportion of the total mass of a tumour, so the selective elimination of this subpopulation with a bioreductive agent would not be expected to result in significant single-agent efficacy. Yet, the experimental observations that vascular shunting and fluctuating blood flow (Chaplin *et al.*, 1987; 1995; Hill *et al.*, 1996; Dewhirst *et al.*, 1992; 1996) causes cell populations to rapidly cycle between the hypoxic and oxygenated tumour compartments, suggests that the clinical opportunities for utilising bioreductive drugs are broader than might have been initially anticipated. It has been suggested that this phenomenon of relatively rapid changes in tumour oxygenation status, underlies the impressive activity of tirapazamine (TPZ) when repeatedly co-administered in a factionation radiation schedule (Brown

and Koong, 1991; Brown and Giaccia, 1994). In marked contrast to HSCs, the use of radiosensitisers in fractionation radiation schedules is associated with loss of activity (Hill, 1986).

1.11 Modifying tumour vasculature to enhance bioreductive drug activation

The combination of HSCs with other treatment modalities that target the aerobic compartment of solid tumours is necessary to adequately treat the whole tumour mass. Alternatively vascular modifiers can be employed, in order to actively render all cells in a tumour mass hypoxic. This can be achieved by selectively inhibiting tumour blood flow through the exploitation of the differences in vascular physiology between normal and neoplastic tissues. For example, the anti-hypertensive agent hydralazine induces selective dilation of normal vasculature, thereby increasing normal tissue blood flow at the expense of the tumour (Chaplin, 1989). Its effectiveness in combination with RSU 1069, EO9 or TPZ has been demonstrated in transplantable tumour models (Chaplin and Acker, 1987; Stratford *et al.*, 1989; Adams *et al.*, 1992; Bibby *et al.*, 1993), but some evidence has suggested that induction of hypoxia is less effective in human xenografts (Rowell *et al.*, 1990). Recent studies have shown that the induction of tumour hypoxia is also seen in spontaneous murine tumours, ruling out the suggestion that reduced tumour blood flow is an artifact of transplantable tumour models (Nordsmark *et al.*, 1996).

The synthetic flavonoid, flavone acetic acid (FAA) induces a profound reduction in blood flow in murine tumours (Hill *et al.*, 1989), which is known to involve the induction of tumour necrosis factor α (TNFα) (Mace *et al.*, 1990) and activation of coagulation (Murray *et al.*, 1989). Clinical trials with FAA alone or in combination with interlukin-2 were disappointing (Olver *et al.*, 1991; Holmlund *et al.*, 1995). A related compound, 5,6-dimethyl xanthenone-4-acetic acid (MeXAA) was found to be 10-15 times more effective than FAA in murine tumours, and is currently in phase I clinical trials (Rewcastle *et al.*, 1991; Cliffe *et al.*, 1994; Wilson and Pruijn, 1995). MeXAA has both vascular and immunological mechanisms of action similar to FAA and causes rapid haemorrhagic necrosis (Zwi *et al.*, 1994; Ching *et al.*, 1994; Laws *et al.*, 1995). MeXAA provides significant enhancement of anti-tumour activity in combination with several bioreductive agents, including TPZ, CI-1010, and SN 23862 (Cliffe *et al.*, 1994; Wilson and Pruijn, 1995; Vincent *et al.*, 1997). In combination with serotonin (5-HT), MeXAA has enhanced antitumour activity (Baguley *et al.*, 1993; Pedley *et al.*, 1996), and the addition of 5-

HT to MeXAA/bioreductive drug combinations can be particularly effective, although therapeutic gain can be dependent upon the class of bioreductive agent used (Simm *et al.*, 1997).

Other strategies such as inhibition of nitric oxide synthase (NOS) to reduce production of the endogenous vasodilator nitric oxide (NO), have also been shown to lower tumour blood flow (Andrade *et al.*, 1992). Nitro-L-arginine, one such NOS inhibitor, can increase radiobiological hypoxia and decrease energy metabolism in certain tumours (Wood *et al.*, 1994a; 1994b), and has been shown to enhance the anti-tumour effects of the 2-nitroimidazole, RB 6145 (Wood *et al.*, 1994b). Alternative approaches to selectively lowering tumour tissue oxygenation include the use of left-shift agents to modify the haemoglobin saturation curve and impede oxygen delivery (Adams *et al.*, 1986).

Novel anti-vascular therapies, such as gene therapy-directed TNFα production, might also be utilised in combination with HSCs (Mauceri *et al.*, 1996). TNFα expression has be specifically localised to the tumour endothelium, and this approach is attractive since endothelium provides a readily accessible target cell population for viral transduction (Jaggar *et al.*, 1997). In principle, selective damage to tumour vasculature through novel endothelial cell-targeted virus-directed cytokine or enzyme/prodrug therapy, may render solid tumours very responsive to secondary treatment with HSCs.

1.12 Bioreductive drugs as hypoxia-activated diffusible cytotoxins

An extension of the idea of targeting hypoxic tumour cells with HSCs, is to exploit the reductive enviroment of a solid tumour to activate HSCs that release oxygen-insensitive diffusible cytotoxins (Wilson, 1992; Denny and Wilson, 1993). Thus tumour hypoxia may provide a unique approach to selectively eliminate a much larger proportion of a tumour mass. Rather than just killing the hypoxic compartment of the tumour, the activated cytotoxin can extend its effects to surrounding tumour tissues of intermediate or normal pO_2 levels. The extent of this "bystander" killing effect can be governed by the reactivity and half-life of the diffusible cytotoxins (Friedlos *et al.*, 1997.) By extending the spatial range of the hypoxia-activated prodrugs, the heterogeneity of cell killing that can arise at intermediate pO_2 levels when conventional HSCs are combined with irradiation, or alternatively through clonogenic loss of bioactivating enzymes, may be effectively overcome (Denny and Wilson, 1993).

Of the five redox centres that have been exploited as bioreducible triggers, only nitro(hetero)aromatics and colbalt(III) complexes have been developed in the context of reductive triggers for diffusible cytotoxins (Denny *et al.*, 1996; Siim *et al.*, 1997). A number of the nitro-triggered bioreductive prodrugs show good efficacy *in vivo*, particularly in combination with anti-vascular strategies (Cliffe *et al.*, 1994; Wilson and Pruijin, 1995; Siim *et al.*, 1997). This class of diffusible nitro-prodrugs also lend themselves to exploitation in combination with gene-directed enzyme/prodrug therapies (GDEPT). Currently *E.coli* nitroreductase (NR) is being developed as an activating enzyme for many of the nitro-prodrugs, but metabolism is not restricted to hypoxic tissues, since it is an oxygen-independent obligate two-electron reductase (Anlezark *et al.*, 1995). Therefore the tissue-specificity of prodrug activation must, like conventional GDEPT, rely upon selective therapeutic gene delivery and/or tissue-specific NR expression. An alternative approach might be to utilise an oxygen-sensitive reductase, perhaps transcriptionally regulated by hypoxia-responsive enhancers/promoters, to effectively exploit the hypoxic compartment of a solid tumour through redox-sensitive, gene-directed enzyme/prodrug therapy. Any inappropriate therapeutic gene delivery to and/or expression would not confer prodrug sensitivity to normoxic tissues. This additional selectivity, ensured by both hypoxia-selective therapeutic gene expression and oxygen-sensitive prodrug activation, could be targeted at the majority of a tumour mass if combined with anti-vascular strategies.

Chapter 2

2. Enzymology of Tirapazamine Metabolism

2.1 Introduction

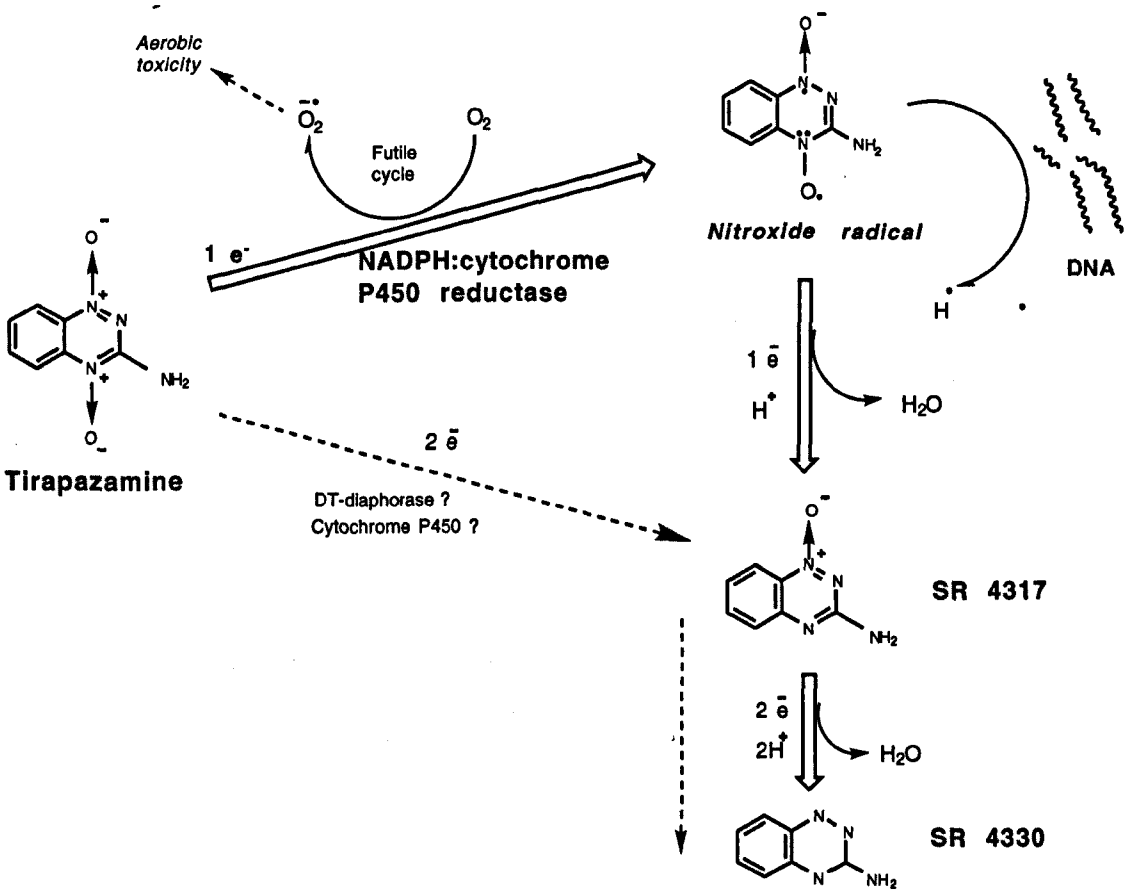
A clearer understanding of the key enzymes involved in the metabolism of tirapazamine (TPZ) is of great importance if this hypoxic cytotoxin is ultimately to be applied to a rationally selected patient population. Its impressive hypoxic selectivity at radiobiologically relevant oxygen tensions and its synergistic interaction with radiation and some chemotherapeutic agents have made it a lead second generation bioreductive compound in the treatment of solid tumours (Workman and Stratford, 1992; Brown, 1993; Koch, 1993; Dorie and Brown, 1993, 1997; Langmuir *et al.*, 1994; Lartigau and Guichard, 1995).

The enzymology of Tirapazamine (TPZ, SR 4233, WIN 59075, 3-amino-1,2,4-benzotriazene 1,4-dioxide, Tirazone™) has been extensively studied in rodents and to a lesser extent in human systems. While it is clear that the initial reductive step in TPZ activation is enzyme-mediated, there is limited consensus in the published literature as to the relative contributions of the cellular reductases involved. Moreover, not only is the importance of sub-cellular localisation for these putative activating reductase(s) far from clear, but their activity profiles *in vivo* are poorly defined. The same might also be said of the potential detoxifying enzymes.

In the presence of certain cellular reductases, TPZ will be subject to one-electron reduction to give the radical anion, which in its protonated form has been identified as a nitroxide radical (Lloyd *et al.*, 1991). This species is unstable in the presence of oxygen and is rapidly back-oxidised to the parent compound with the concomitant production of significantly less cytotoxic superoxide radical species (Lloyd *et al.*, 1991). This "futile cycling" in the presence of oxygen may account for the less severe oxic cellular toxicity of TPZ, probably due to the abundance of cellular reactive oxygen detoxifying mechanisms, most notably the superoxide dismutase(s) and catalase combination. In contrast, the absence of oxygen facilitates a TPZ radical mediated oxidising reaction with bio-molecules e.g. hydrogen abstraction from DNA (Laderoute *et al.*, 1988; Baker *et al.*, 1988) resulting in the formation of the non-toxic two-electron reduction product, SR 4317. An oxidative mechanism of free radical attack, rather than

TPZ-nucleic acid base adducts, is most likely, since TPZ exposure results in DNA double strand breaks, whilst virtually no covalent binding of this drug to DNA or other cellular macromolecules occurs.

Figure 1: Proposed mechanism of hypoxia selectivity of tirapazamine.



Consistently, electron spin resonance spectrometry (esr) evidence has clearly shown that a TPZ nitroxide radical anion can be formed, although the authors did not speculate as to the oxidising capability of this radical (Lloyd *et al.*, 1991). The protonated neutral form of a TPZ nitroxide radical is presumed to be the reactive species, although there is no direct evidence for this. It has been further demonstrated that TPZ interaction with DNA results in formation of phosphoglycolates; fragments of damaged deoxyribose moieties (Jones & Weinfield, 1996). Since this requires the addition of oxygen it is concluded that TPZ acts in a dual fashion, firstly by

a TPZ free radical mediated attack on ribose to generate a C-4' free radical, following which unmetabolised TPZ then donates oxygen to this sugar radical by an oxygen atom transfer process. This produces an oxy-radical that decomposes to the phosphoglycolates observed. A further complication to this mechanism is evidence that the protonated neutral TPZ radical may also decompose by liberating a hydroxyl radical in keeping with direct formation of the mono-N-oxide metabolite (Daniels & Gates, 1996). These authors also showed that TPZ-mediated strand breakage is DNA non-sequence specific consistent with involvement of a highly reactive, non-selective cleaving agent such as the hydroxyl radical. Using esr spectrometry in combination with DMPO as a spin trapping agent we have shown formation of a hydroxyl radical following anaerobic incubation of TPZ in several different human tumour xenograft microsomes (Patterson *et al.*, 1998). Whatever the precise nature of the TPZ free radical it is the primary reactive species responsible for the cytotoxicity of this agent.

The stoichiometry observed in mouse liver microsomes and human cell lines is reported to be 1.7 - 2.5 moles TPZ consumed for each mole of SR4317 formed, leaving 30 to 60% of starting material apparently unaccounted for (Walton and Workman, 1990; Walton *et al.*, 1992; Siim *et al.*, 1996). Very little, if any, four-electron reduction product, SR4330, is generated by rodent liver microsomal metabolism (Walton *et al.*, 1992) although rat DT-diaphorase appears particularly efficient at generating this product, and perhaps even a six-electron reduction product (Riley and Workman, 1992). Although some material can be accounted for in an unidentified HPLC peak (Baker *et al.*, 1988; Laderoute *et al.*, 1988; Walton and Workman, 1990) the observed stoichiometry is largely unexplained. Very little ^{14}C-TPZ ($\leq$ 0.1 %) is found associated with DNA (Laderoute *et al.*, 1988), and plasma protein binding in mouse and human pharmacokinetic studies is only $\approx$ 10 and 19% of total TPZ, respectively (Robin *et al.*, 1995). Despite this gap in our understanding, the rate of SR 4317 formation has been related to cytotoxicty in a number of studies (Biedermann *et al.*, 1988; Costa *et al.* 1989) but not in others (Siim *et al.*, 1996). The high proportion of unrecoverable TPZ during reductive metabolism studies suggest that other highly reactive TPZ species are generated. These are most likely due to fragmentation of the chromophore producing (probably small) reactive metabolites that hitherto have evaded detection. The possible contribution of unknown reactive species to TPZ cytotoxicity should not be underestimated since a mechanism of action based on the nitroxide free radical is far from certain. Alternative possibilities have been suggested; for example, in principle TPZ could be enzymatically activated through a ring opening mechanism producing an unstable intermediate that can fragment, with the theoretical formation of a cytotoxic phenyldiazonium ion or an oxygen-inhibited phenyl radical (Laderoute *et al.*, 1988; Ara *et al.*, 1994). Whatever the underlying mechanism for DNA damage, there is a clear relationship between the rate of

formation of single-strand breaks (Siim *et al.*, 1996), the final extent of unrepaired chromosome aberrations (Wang *et al.*, 1992) and hypoxic cytotoxicity. Thus, the level of hypoxia (Koch, 1993; Lartigau and Guichard, 1995), the ability of cells to repair DNA damage (Keohane *et al.*, 1990; Biedermann *et al.*, 1991; Olive, 1995), and the expression of cellular reductases, will all influence the therapeutic selectivity of TPZ.

In order to assess the role of the various reductase enzymes in a physiologically relevant context, activities of four major reductase enzymes were determined for a panel of human lung and breast cancer cell lines *in vitro*, and related to their hypoxic and aerobic sensitivity to TPZ, and the ability of cell lysates to metabolise TPZ to its stable 2-electron reduced product SR4317.

The panel of breast and lung cancer cell lines were chosen to represent the range of biological characteristics found clinically. The breast cell lines expressed a variable spectrum of growth factor and hormone receptors. SKBr3 (Trempe and Fogh, 1973) over-expresses c-erbB2 and topoisomerase IIα, MDA-468 (Cailleau *et al.* 1974) and MDA-231 (Cailleau *et al.* 1978) are oestrogen receptor negative (ER -ve) and epidermal growth factor receptor positive (EGFR +ve), while T47D (Freake *et al.* 1981), MCF-7 (Soule *et al.* 1973) and ZR-75 (Engel *et al.* 1978) are ER +ve and EGFR -ve. ZR-75 is also progesterone receptor positive (PGR +ve), while MDA-231 is positive for c-erbB3. HBL-100 is a non-tumourogenic, SV40 large T-antigen transformed cell line that is ER -ve (Polanowski *et al.* 1976) The lung cancer cell lines were representative of the major histological subtypes and include four small cell lung cancer (SCLC) cell lines, two bronchioloalveolar cell lines and two adenocarcinoma cell lines (Carmichael *et al.* 1988).

Where possible, cell lines were established as xenografts in athymic nude mice and allowed to grow to ~ 300mm^3 before being surgically removed and snap frozen in liquid N_2 for further analysis. Identical analyses of reductive enzyme activities were performed as for the *in vitro* lysates.

2.2 Methods

Chemicals. Tirapazamine, SR4317 and SR4330 were obtained from Sterling Winthrop or synthesised in house (Dr. M. Jaffar) using previously described methods (Seng and Ley, 1972). NADPH was purchased from Boehringer Mannheim (Lewes, UK). HPLC grade methanol was purchased from Merck (Lutterworth, UK). All other reagents were of analytical grade and were

purchased from Sigma (Poole, UK) or Aldrich. Tissue culture medium was obtained from ICRF (Clare Hall Labs., UK.) and Fetal calf serum from Sigma.

Cells and culture. Table 1 lists the 22 cell lines used in this work. All cell lines were maintained in exponential growth phase by routine passaging. Most cell lines were grown in RPMI-1640, except for SKBr3 and SK-MES which were maintained in DMEM, and CALU-3 and LDAN which were maintained in a 1:1 mixture of DMEM:Hams F-12. (All media were prepared at ICRF, Clare Hall.) Media was supplemented with 10% (v/v) fetal calf serum and 2mM glutamine. No antibiotic supplements were used. Cells were grown at 37°C in 100% humidified incubator with a gas phase of 5% CO_2, 21% O_2. Cultures were routinely screened and found free of *mycoplasma*.

***In vitro* quantification of TPZ sensitivity.** A panel of seven breast and fifteen lung cell lines were assessed for their sensitivity to TPZ in two sets of experiments. Cells were either exposed to drug for 3h under aerobic or hypoxic conditions and allowed to grow in 0.4 ml fresh media for a total of 96h, or grown in air (final volume 0.2 ml) for 96h in the constant presence of TPZ. Dose response curves were determined using the MTT proliferation assay which is based on the ability of viable cells to convert a soluble tetrazolium salt, MTT, into purple formazan crystals (Mossman 1983). The optical density is proportional to the number of viable cells, although the absolute metabolism varies slightly between cell lines, as the conversion of MTT to formazan depends on the level of mitochondrial dehydrogenase activity in each cell line (Carmichael *et al.* 1987). Previous experiments have demonstrated that cellular sensitivity to bioreductive agents, whether assessed by clonogenic survival or the MTT assay, is similar and the two assay methods correlate well (Stratford and Stephens 1989). Cells are seeded at 10^3 to 10^4 cells per well (depending on the doubling time of the cell line in question) in either 24- or 96-well format and allowed to attach for 3h prior to drug exposure. After 4 days, MTT (0.2 mg/ml) was added to a final concentration of 40 µg/ml and cells were incubated for a further 4h. Culture medium and unconverted MTT were removed and the formazan crystals dissolved in 200 µl DMSO, 25 µl glycine buffer, pH 10.5 (Plumb *et al.*, 1989). Following 5 min vigorous shaking, optical density at 540 nm was measured on a multi-well spectrophotometer (Titretek Multiscan Plus MKII, Flow laboratories). Values of IC_{50} (the concentration of drug required to reduce optical density by 50% compared to untreated controls) were determined from the individual survival curves of independent experiments (Deltasoft software; Biometallics Inc., Princeton, USA). Mean IC_{50} values (± s.d.) were derived from at least 6 independent experiments conducted on different days.

Preparation of *in vitro* cell lysates. Cells in exponential growth phase were washed twice in ice cold PBS and harvested using a sterile cell scraper. Cells were resuspended in 1.5ml ice cold hypotonic nuclear buffer A (10mM HEPES/KOH, 1.5mM $MgCl_2$, 10mM KCl, 0.05mM DTT, pH 7.4) and allowed to stand for 10 min at 4°C. Suspensions were sonicated using a MSE Soniprep 150 for 3 x 5 sec. at a nominal frequency of 23kHz and an oscillation amplitude between 5 and 10 μm. Samples were placed on ice between each sonication. The suspensions were allowed to stand on ice a further 10 min. and then were centrifuged at 7800g for 15 min at 4°C. Protein content of the cell lysates was determined using the Bio-Rad protein dye binding assay (Bradford 1976) and quantified against high grade BSA protein standard. All samples were stored in liquid N_2 until required (< 6 weeks).

Preparation of human cell line xenografts. All of the cell lines that would form viable xenografts when inocculated subcutaneously in nude mice were profiled. In some cases (e.g. MCF-7, ZR-75) it was necessary to support xenograft growth with a subcutaneous 90 day release oestrogen (1.7mg) implant. Cells were harvested in exponential growth phase, washed twice in cold PBS and resuspended at $2x10^7$ cells/ml in cold serum free RPMI. Using pre-cooled glass pipettes, the cell suspension was mixed with an equal volume of pre-cooled matrigel and 100μl aliquotes (10^6 cells) were inoculated subcutaneously into the hind leg of female nude mice. Growth was monitored weekly, and xenograft tissues were removed at ≈ $400mm^3$ and snap frozen in liquid N_2. Samples were homogenised and excess debris was removed with an initial 3000g spin (4°C). S-9 fractions were prepared, assayed and stored in an identical manner to the *in vitro* lysates.

NADPH:cytochrome P450 reductase activity. P450 reductase activity was determined spectrophotometrically by monitoring the ability of the cell lysates to catalyse the NADPH-dependent reduction of cytochrome c. (Patterson *et al.* 1995b). Each incubation comprised 400 μl cytochrome c (final concentration 50μM), 100 μl 10mM KCN (final concentration 1 mM) and 100-300 μg lysate protein (50-100 μl) made upto 0.98ml with 100mM potassium phosphate buffer, pH 7.4. The reaction was equilibrated to 37°C and initiated by addition of 20 μl 10mM NADPH to the test cuvette (final concentration 200μM) and the rate of reduction of cytochrome c was monitored at 550nm for 3 min against a blank without NADPH. Initial rates of reaction were based upon an extinction coefficient of 21.0 mM^{-1} cm^{-1}. P450 reductase activity was determined for at least 5 independent lysates, harvested on different days and the results were expressed as nmol cyt.c reduced per minute per mg of lysate (S-9) protein.

NADH:cytochrome b_5 reductase activity. B_5 reductase activity was determined spectroscopically as the *para* -hydromecuricbenzamide (*p*-HMB) -inhibitable component of the NADH supported reduction of cytochrome c (37°C) (Barham *et al.*, 1996).

NAD(P)H: DT-diaphorase activity. DT-diaphorase activity of the cell lysates was determined spectrophotometrically as the dicumoral-inhibited component of NADH-dependent reduction of cytochrome c when supported by menadione (Patterson *et al.*, 1994; Robertson *et al.*, 1994).

Cytochrome P450 isozyme activity. Cytochrome P450 (CYP P450) activity determinations were carried out by Prof. Laurence Patterson (Dept. Pharmaceutical Sciences, De Montford University, Leicester).The assay is based upon the ability of specific CYP P450 isozymes to catalyse the O-dealkylation of specific alkyloxyresorufin analogues to produce the highly fluorescent compound resorufin (Burke *et al.*, 1994). Methoxyresorufin O-demethylation (MROD) and ethoxyresorufin O-deethylation (EROD) are probes for two closely related forms of human and rat CYP1A1 and CYP1A2 respectively. Pentoxyresorufin O-dependentylation (PROD) is a probe for CYP2B activity, whilst benzyloxyresorufin O-debenzylation is specific to the CYP3A family. The assay used is a modification of the method used by Burke and Mayer (1975). The reaction was carried out under yellow light to prevent photo-decomposition of the alkylquinolinol.The reaction mixture contained 5µM alkoxyresorufin substrate (5µl of a 1mM solution in DMSO), 100µM sodium phosphate buffer (pH 7.6), 10µM dicoumarol (5µl of a 2mM solution in DMSO) and 250µM NADPH (5µl of a 50mM solution in sodium phosphate buffer). This was pre-incubated for 2 minutes at 37°C in the housing unit of a Perkin-Elmer Model LS-5B luminescence spectrophotometer. The reaction was initiated by adding 50µl of S-9 (prepared from cell line or xenograft). The total reaction volume was 1ml. The excitation wavelength was 530nm and emission was monitored at 585nm (slit width 5nm). Rate of formation of resorufin was calculated from an authentic resorufin standard. Positive control reactions were carried out as above but with the S-9 proteins replaced with liver microsomes prepared from untreated rat (50µl of 8.9 mg/ml microsomal protein) for MROD, EROD and BROD. Microsomal proteins from the liver of PB-treated rat (50µl of 1.65 mg/ml micrsomal protein) were used to assay for PROD.

Analysis of total intracellular glutathione. Total glutathione (GSH) was determined as the NADPH dependent reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) when supported by excess exogenous glutathione reductase (30°C) (Griffith, 1980). DTNB is converted to a yellow compound, 2-nitro-5-thiobenzoic acid (DTTB), the formation rate of which can be monitored spectroscopically at 412nm. The formation rate of DTTB is proportional to the

concentration of (GSH) in the sample, and values can be interpolated via an authentic GSH standard of known concentration. Values were expressed as nmol GSH / 10^6 cells.

Western immunoblot analysis. Samples of cells harvested for enzyme assays were washed in cold PBS buffer containing 1mM phenlymethylsulphonyl fluoride, 1mM benzamide, 50 μg/ml leupeptin and 50 μg/ml soyabean trypsin inhibitor. Cells were lysed in 1 ml 2% SDS, plus inhibitors in PBS at 65°C for 5 min. DNA was broken up by passing up and down a fine gauge needle. Samples were stored at -20°C. Proteins were subsequently resolved by 10% SDS-polyacrylamide gel electrophoresis, and proteins on the gel were transferred overnight to a nitrocellulose hybridization transfer membrane. The membrane was washed 3 x 15 min with blocking buffer (20mM Tris-HCl / pH7.5, 0.9% NaCl, 0.5% Tween 20, 1% low fat Marvel), and incubated for 60 min with specific anti-human P450 reductase rabbit antibody (dilution 1:500). After washing, horseradish peroxidase-conjugated goat anti-rabbit antibody (dilution 1:5000) was added and incubated for 30 min. All antibodies were diluted in blocking buffer. The membrane was developed using an enhanced chemo-luminescence western blotting detection kit (Amersham, U.K.).

Metabolism of tirapazamine by *in vitro* cell lysates. Incubations were carried out under nitrogen at 37°C. The final incubation volume of 500μl comprised 100μl cell lysate (max. final concentration 1.5 mg/ml), 100μl NADPH (final concentration 1mM), 20μl TPZ (final concentration 2mM) and 280μl of 200mM potassium phosphate buffer, pH 7.4. The reaction was initiated by addition of substrate and stopped after 40 min by transferring 2 x 200μl aliquots into 400μl methanol and 50μl internal standard (0.4 mg/ml 4-nitroquinoline N-oxide in 20% (v/v) ethanol). Supernatants were prepared and injected onto the HPLC for analysis. Three lysate preparations for each cell line were incubated, each in duplicate, with duplicate analyses. Formation of SR4317 was linear for at least 40 min and up to a protein concentration of 1.5 mg/ml, determined using SKBr3 lysates, which have the highest endogenous P450 reductase activity amongst the panel of cell lines.

HPLC. Concentrations of SR4317 and SR4330 in incubation samples were determined by isocratic reverse phase HPLC (Walton and Workman 1990). Chromatography was performed using a waters μBondpak phenyl 4μ radial compression cartridge in a Waters Radial Compression Module, and protected with a phenyl guard column. The mobile phase consisted of 32% methanol in water delivered at a flow rate of 3 ml/min. Detection was at 267 nm. Approximate retention times were 2.7, 4.6, 5.2 and 8.4 min for tirapazamine, SR4330, SR4317 and 4-nitroquinoline N-oxide respectively. Concentrations of metabolites were calculated from peak height ratios and

compared with calibration curves (0-500μM) prepared by spiking lysate preparations with known amounts of metabolite.

Data analysis. P450 reductase activity measurements were determined for each of the cell lysates and compared with the IC_{50} values derived for each cell line under hypoxic and aerobic conditions. The abilities of the different cell line lysates to metabolise TPZ to its reduced product SR4317 under hypoxic conditions were determined. The relationship between TPZ sensitivity, P450 reductase, B_5 reductase, DT-diaphorase activities and SR4317 formation velocity was assessed. The datawere analysed using the standard model for a linear functional relationship with sampling errors in both variables. Briefly, the data were logarithmically transformed and the pooled variance of each data set was calculated. For statistical analysis of any two data sets, the model is fitted to the observations by the method of weighted least squares, each sample mean being weighted in direct proportion to the sample size. The statistical goodness-of-fit for any two data sets was tested by calculating the weighted mean-square deviation of the observations for mean xn and mean yn from the fitted model, and comparing this mean-square with the pooled variance within samples by a variance-ratio test. The statistical significance of the estimate of the slope of the straight line of best fit was tested by a students t-test.

2.3 Results

Analysis of the inter-relationships between hypoxic and aerobic TPZ sensitivity, a complement of cellular reductase activities, intracellular glutathione and SR4317 formation velocity for the *in vitro* human lung and breast cell lines was conducted. The characterisation of endogenous reductase activities found in the *in vitro* cell line panel is summarised in table 1. No statistically significant relationship was found for TPZ sensitivity and any of the parameters in the lung cell lines. In contrast, there was a clear relationship between NADPH:P450 reductase activity, sensitivity to TPZ exposure and SR4317 formation velocity in this panel of breast cell lines (table 2) (Patterson *et al.* 1995b).

Table 1. *In vitro* determinations of key enzymes in a panel of human breast and lung cell lines.

Cell line	Tissue type	Characteristics	NADPH: P450 reductase	NADH: B_5 reductase	NAD(P)H: DT-diaphorase	Cytochrome P450 3A	Glutathione
			nmol cyt. c reduced/min/mg	nmol cyt. c reduced /min/mg	nmol cyt. c reduced/min/mg	BROD oxidation pmol/min/mg	nmol GSH /10^6 cells
SKBr3	adenocarcinoma	↑ c-erbB2,top IIα	39.8 ± 3.2	59.7 ± 7.3	1870 ± 480	1.89	12.2
MDA-468	adenocarcinoma	EGFR+ve	20.9 ± 1.8	38.9 ± 8.3	13 ± 2	1.71	16.4
T47D	adenocarcinoma	ER+ve	17.2 ± 3.3	50.4 ± 6.5	46 ± 15	n.d.	13.0
MCF-7	adenocarcinoma	ER+ve	18.7 ± 3.4	61.3 ± 3.6	709 ± 442	< 0.1	30.5
ZR-75-1	adenocarcinoma	ER & PGR+ve	11.2 ± 2.3	109 ± 11	2290 ± 650	< 0.1	11.8
MDA-231	adenocarcinoma	EGFR+ve	6.7 ± 2.0	49.8 ± 3.0	12 ± 6	< 0.1	9.0
HBL-100	non-tumourigenic	SV40 transformed	8.8 ± 0.1	102 ± 9	< 1	n.d.	-
A549	NSCLC	adenocarcinoma	22.0 ± 2.7	51.6 ± 3.4	5930 ± 1500	1.38	40.8
CALU-3	NSCLC		21.5 ± 0.8	83.0 ± 7.6	10 ± 4	< 0.1	-
SK-MES	NSCLC		19.2 ± 1.5	37.1 ± 12	362 ± 88	< 0.1	-
NCI-H 322	NSCLC	bronchio-alveolar	18.4 ± 3.2	60.7 ± 24	5030 ± 1632	0.99	41.2
NCI-H 358	NSCLC	bronchio-alveolar	17.7 ± 1.0	39.0 ± 7.2	3290 ± 196	< 0.1	14.1
NCI-H 460	NSCLC	large cell	17.6 ± 2.3	38.7 ± 4.7	5340 ± 989	< 0.1	33.0
NCI-H 522	NSCLC	adenocarcinoma	15.4 ± 1.9	78.4 ± 1.6	240 ± 79	< 0.1	4.6
LDAN	NSCLC		9.2 ± 0.4	35.9 ± 4.6	403 ± 97	< 0.1	-
NCI-H 647	NSCLC	adenosquamous	9.1 ± 1.0	49.7 ± 5.0	5140 ± 781	0.94	33.4
NCI-H 226	NSCLC	squamous carcin.	7.3 ± 1.0	81.5 ± 4.8	1040 ± 291	n.d.	17.0
NCI-H 841	SCLC	variant	31.6 ± 5.0	71.3 ± 2.6	450 ± 81	n.d.	4.0
NCI-H 249	SCLC		27.1 ± 4.5	92.2 ± 16	5 ± 3	< 0.1	4.2
NCI-H 69	SCLC		16.6 ± 3.4	61.0 ± 10	403 ± 47	n.d.	-
NCI-H 417	SCLC	variant	13.1 ± 1.7	-	120 ± 48	n.d.	7.1
NCI-H 526	SCLC	variant	8.1 ± 0.7	-	19 ± 10	n.d.	-

Table 2: Sensitivity of a panel of human cancer cell lines to aerobic and hypoxic TPZ exposure, and the rates of hypoxic TPZ metabolism to the two-electron reduction product, SR 4317.

Cell line	Tirapazamine IC_{50} (μM) ± s.d.				SR4317 formation velocity
	96h aerobic	3h aerobic	3h hypoxic	O_2:N_2 differential	nmol formed min^{-1} mg^{-1}
SKBr3	10.4 ± 0.3	161 ± 48	5.1 ± 2.0	**29.8**	22.4 ± 3.4
MDA-468	20.1 ± 4.1	181 ± 76	7.3 ± 2.1	**23.4**	11.0 ± 1.9
T47D	20.9 ± 5.2	294 ± 68	12.7 ± 2.0	**23.3**	13.2 ± 2.7
MCF-7	23.1 ± 2.9	546 ± 61	15.0 ± 3.9	**37.1**	10.2 ± 0.9
ZR-75-1	26.6 ± 1.6	1235 ± 502	18.9 ± 2.4	**65.8**	9.1 ± 1.4
MDA-231	35.4 ± 4.1	302 ± 65	22.8 ± 4.0	**15.0**	3.7 ± 0.7
HBL-100	24.0 ± 2.0	550 ± 56	11.7 ± 2.2	**46.9**	4.1 ± 0.4
A549	21.5 ± 5.4	247 ± 44	7.4 ± 0.5	**33.2**	15.1 ± 2.4
CALU-3	-	-	-	-	5.2 ± 0.7
SK-MES	-	396 ± 84	18.4 ± 4.2	**21.5**	16.1 ± 2.4
NCI-H 322	62.4 ± 7.2	980 ± 24	54.3 ± 1.7	**18.0**	13.6 ± 1.6
NCI-H 358	120 ± 32	1231 ± 62	48.1 ± 5.6	**25.6**	7.3 ± 0.7
NCI-H 460	13.5 ± 1.3	110 ± 10	4.2 ± 2.0	**26.2**	11.8 ± 2.3
NCI-H 522	32.3 ± 1.3	141 ± 12	8.9 ± 0.6	**15.8**	9.3 ± 3.0
LDAN	-	-	-	-	14.5 ± 2.9
NCI-H 647	16.7 ± 1.3	135 ± 22	6.3 ± 2.0	**21.3**	8.8 ± 2.5
NCI-H 226	56.4 ± 10	941 ± 189	22.8 ± 4.6	**41.4**	5.5 ± 2.1
NCI-H 841	10.2 ± 1.0	-	-	-	9.3 ± 0.1
NCI-H 249	14.9 ± 1.3	-	-	-	7.7 ± 1.4
NCI-H 69	-	-	-	-	9.3 ± 0.8

Intrinsic sensitivities of human cell lines to the cytotoxic effects of aerobic and hypoxic tirapazamine exposure. The toxic effects of TPZ on the panel of cell lines was determined in two sets of experiments. Firstly, by exposing cells to drug for 3h under aerobic or hypoxic conditions, and secondly by growing cells in air for 96h in the continual presence of TPZ. Cytotoxicty was assessed by the MTT proliferation assay and typical survival curves, derived from individual experiments as given in figure 2.

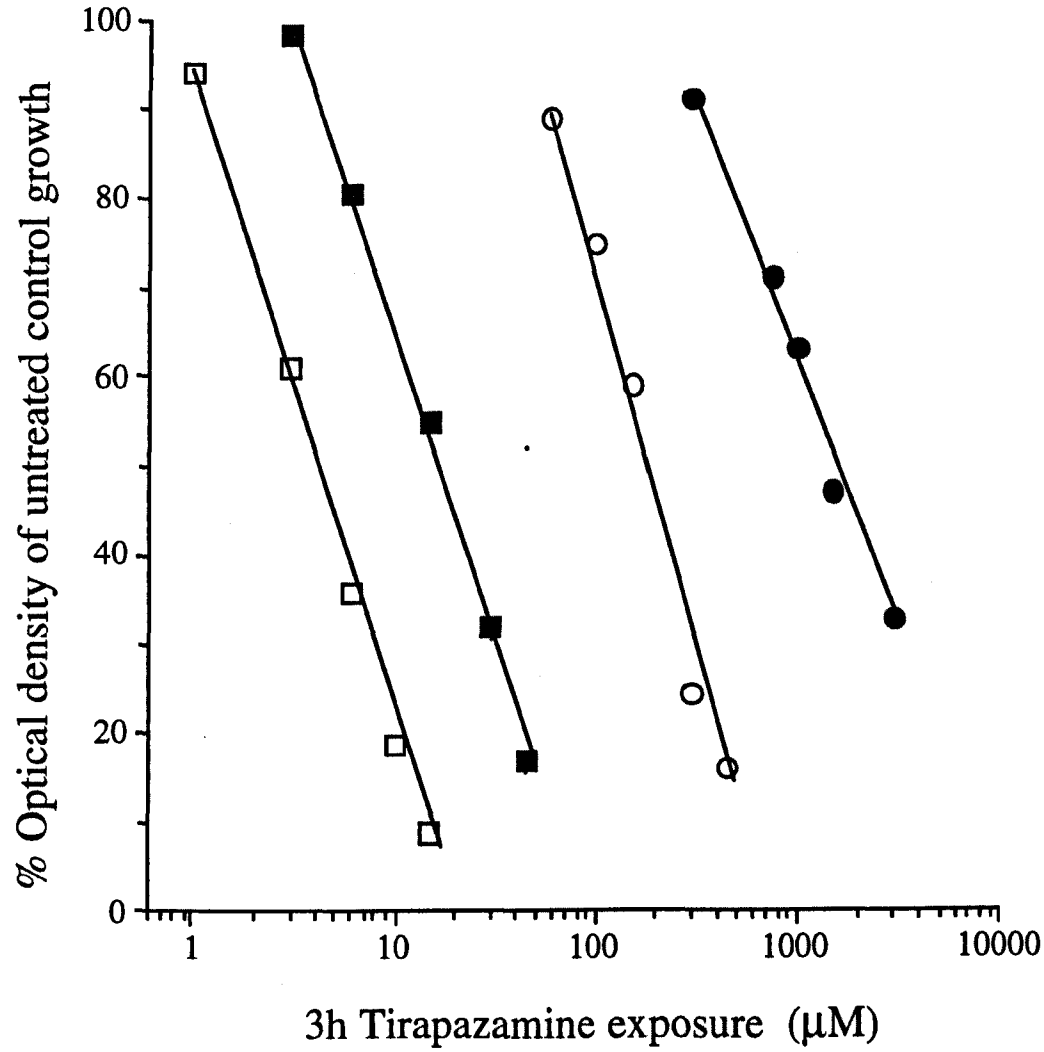


Figure 2: Representative dose-response curves for the P450R-rich SKBr3 cell line (open symbols) and P450R-poor ZR-75 cell line (closed symbols) for 3h aerobic (circular symbols) or 3h hypoxic (square symbols) exposure to tirapazamine.

From these dose-response curves, values of IC_{50} (concentration of drug that will inhibit growth by 50%) can be derived. The data demonstrates the large increase in toxicity that results from TPZ exposure under hypoxic conditions, and also shows the variation in the absolute potency of the drug in air and hypoxia. The spectrum of sensitivity to TPZ is exemplified by the 12-fold range in aerobic and hypoxic IC_{50}s seen across the cell line panel (table 2).

P450 reductase activity correlates with sensitivity to TPZ in the human breast but not lung cell line panels. A 6-fold range in endogenous P450 reductase activity was found across the panel of 22 cell lines. This variation was narrower (3-fold) in the lung cell lines. The collective data were in broad agreement with the spectrum of expression observed for the NCI panel of 69 cell lines, which includes a range of tissue types (Fitzsimmons *et al.*, 1996). Statistical analysis of the data revealed that the IC_{50} values derived from acute 3h exposures to TPZ under hypoxic conditions correlated strongly with the independently determined P450 reductase activities for the breast adenocarcinoma cell lines (slope value = -1.1 ±0.17; $P > 10^{-10}$). The SV40 T antigen immortalised cell line, HBL-100, was atypically sensitive to TPZ and was excluded from the final breast adenocarcinoma cell line analysis. In contrast, aerobic toxicity following a 3h exposure does not show such a statistical dependency (slope value = -1.34 ±0.84; P=0.19) (figure 3). However the relationship under aerobic conditions was apparent following chronic (96h) exposure (slope value = - 1.6 ±0.3; P=0.0057) (figure 4).

Eight of the NSCLC cell lines (A549, SK-MES, H322, H358, H460, H522, H647, H226) were also evaluated for acute TPZ sensitivity. No correlation was found with intracellular P450 reductase activity under either 3h hypoxic (P=0.34) or 3h aerobic (P=0.71) exposure conditions (figure 5). Consistently, no relationship was seen for 96h aerobic exposure to TPZ (P=0.81) (figure 6).

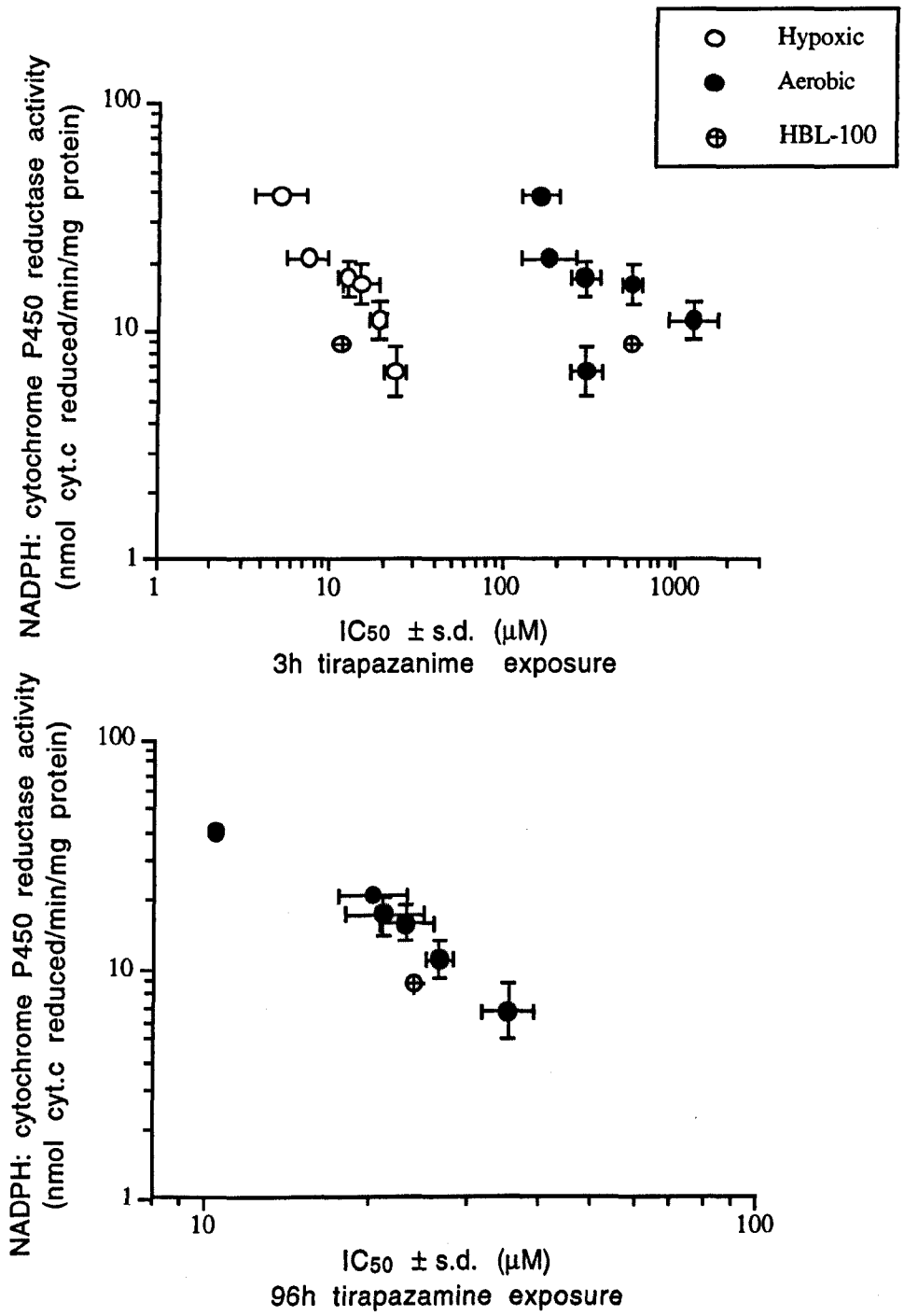


Figure 3 & 4: Human breast cell lines. Relationships between P450 reductase activity and 3h or 96h tirapazamine exposure under either aerobic (●) or hypoxic (O) conditions.

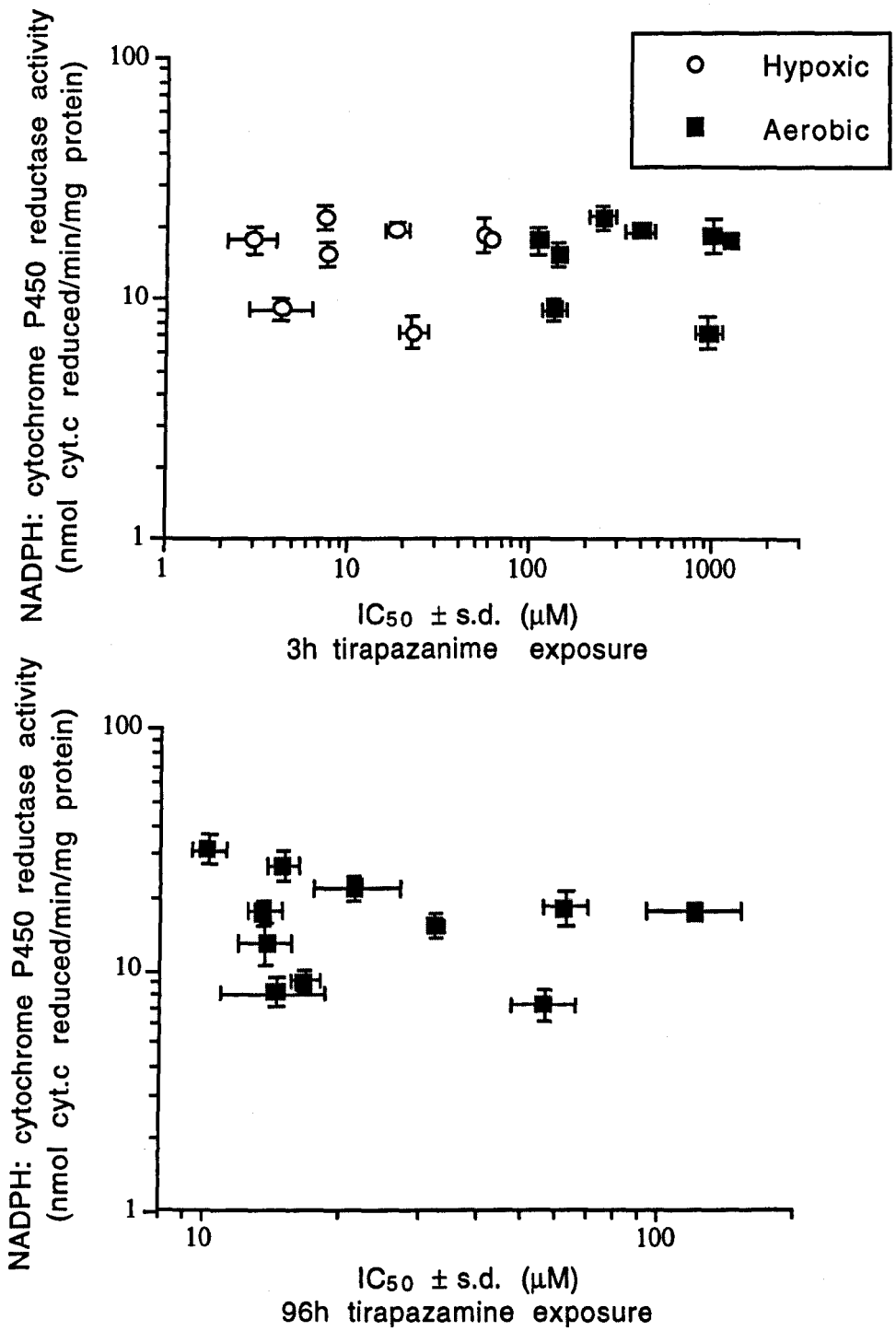
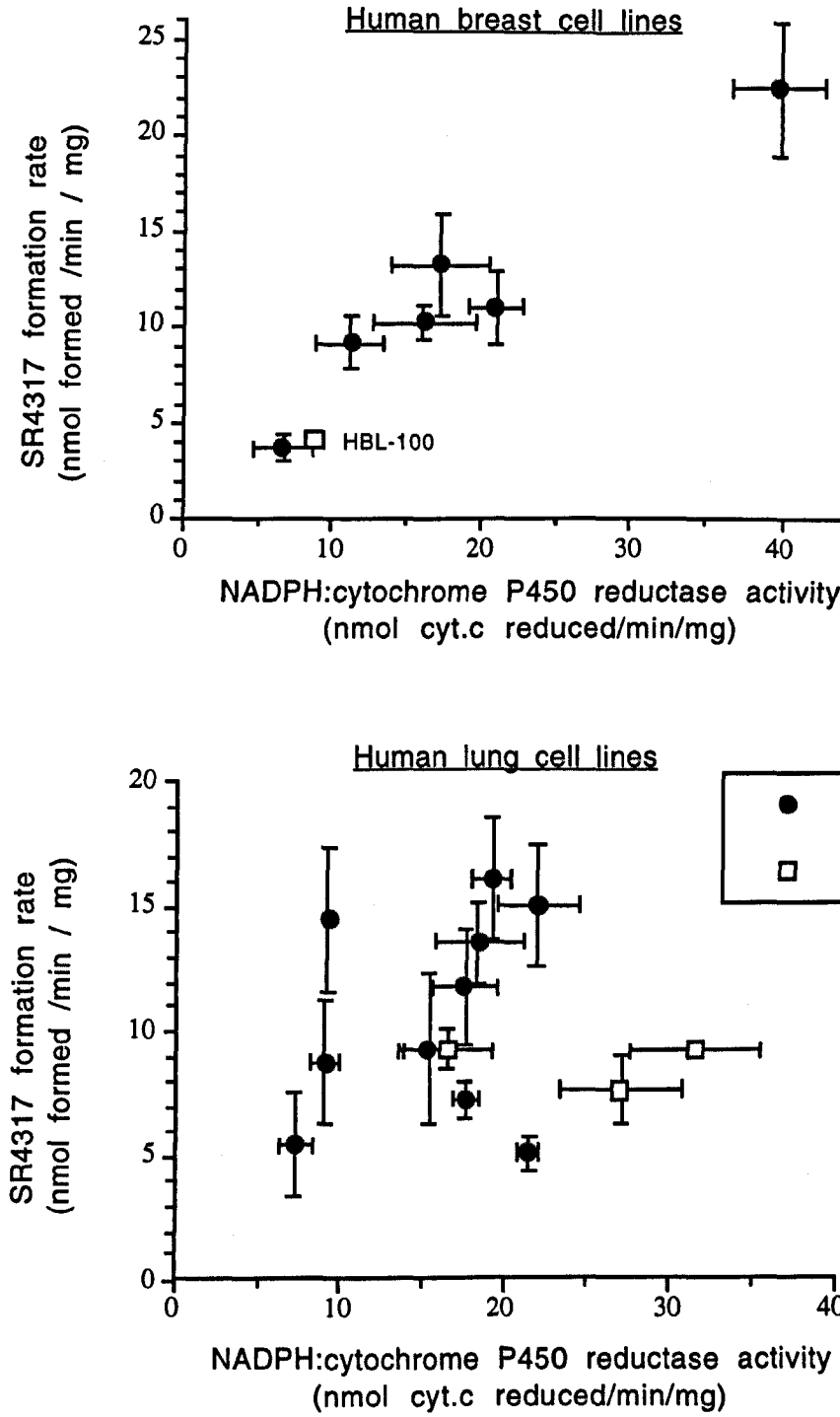


Figure 5 & 6: Human lung cell lines. Relationships between P450 reductase activity and 3h or 96h tirapazamine exposure under either aerobic (●) or hypoxic (O) conditions.

P450 reductase activity correlates with SR4317 formation velocity in the human breast but not lung cell line panels. In order to confirm whether P450 reductase is a major factor contributing to the metabolism of TPZ in the breast cell lines, the rate at which cell lysates reduce TPZ to its 2-electron reduced product SR4317, was measured under hypoxic conditions. The structures of these compounds are given in figure 1. No formation of the deoxygenated 4-electron reduced product, SR4330, was detected in these experiments. The rates of formation are given in table 2 and a plot of P450 reductase activity in the breast cell lines versus the rate of SR4317 formation velocity cataysed by lysates of each cell line, with NADPH provided as the electron source, is given in figure 7. With respect to the breast cell lysates, it is clear that a strong correlation exists between P450 reductase activity and SR4317 formation, with higher values of enzyme activity resulting in greater rates of metabolism (slope = 1.10 ±0.23; P=0.009). Interestingly in the HBL-100 immortalised cell line, reduction of TPZ to SR4317 was consistent with the levels of P450 reductase expression, indicating that the hypersensitivity of this cell line was not a consequence of elevated reductive metabolism of the drug. This suggests that HBL-100 differs from the six adenocarcinoma cell lines in its ability to respond to the TPZ induced DNA damage.

The relationship found in the 7 breast cell lines is not apparent in the panel of 13 lung cell line lysates (P=0.94), with up to 5-fold differences in SR4317 formation velocities for cell lines of similar P450 reductase activities (figure 8). This suggests that other enzymes besides P450 reductase are contributing to the overall reductive metabolism of TPZ in this *in vitro* model.

However, although conversion of TPZ to its one-electron reduced product is an enzyme mediated process under hypoxic conditions, subsequent conversion to SR4317 is a chemical process. Thus measurement of formation of SR4317 is an indirect measure of "toxic" radical formation. Therefore, in the breast cell lines (but not the lung), one would anticipate that the formation of SR4317 by cell lysates should correlate with the IC_{50} for TPZ under hypoxic conditions, which is indeed the case (slope = -0.93 ±0.30; P=0.036) (figure 9). No such correlation is seen in the NSCLC and SCLC panel (P=0.87) (figure 10). When similar metabolism experiments were carried out in air, no SR4317 formation was detected, indicating that the obligate two-electron reductase, NAD(P)H dependent DT-diaphorase, is not measurably contributing to drug metabolism.



Figures 7 & 8: Human breast & lung cell lines. Relationships between endogenous P450 reductase activity and the rate of hypoxic metabolism of TPZ to its two-electron reduction product SR 4317.

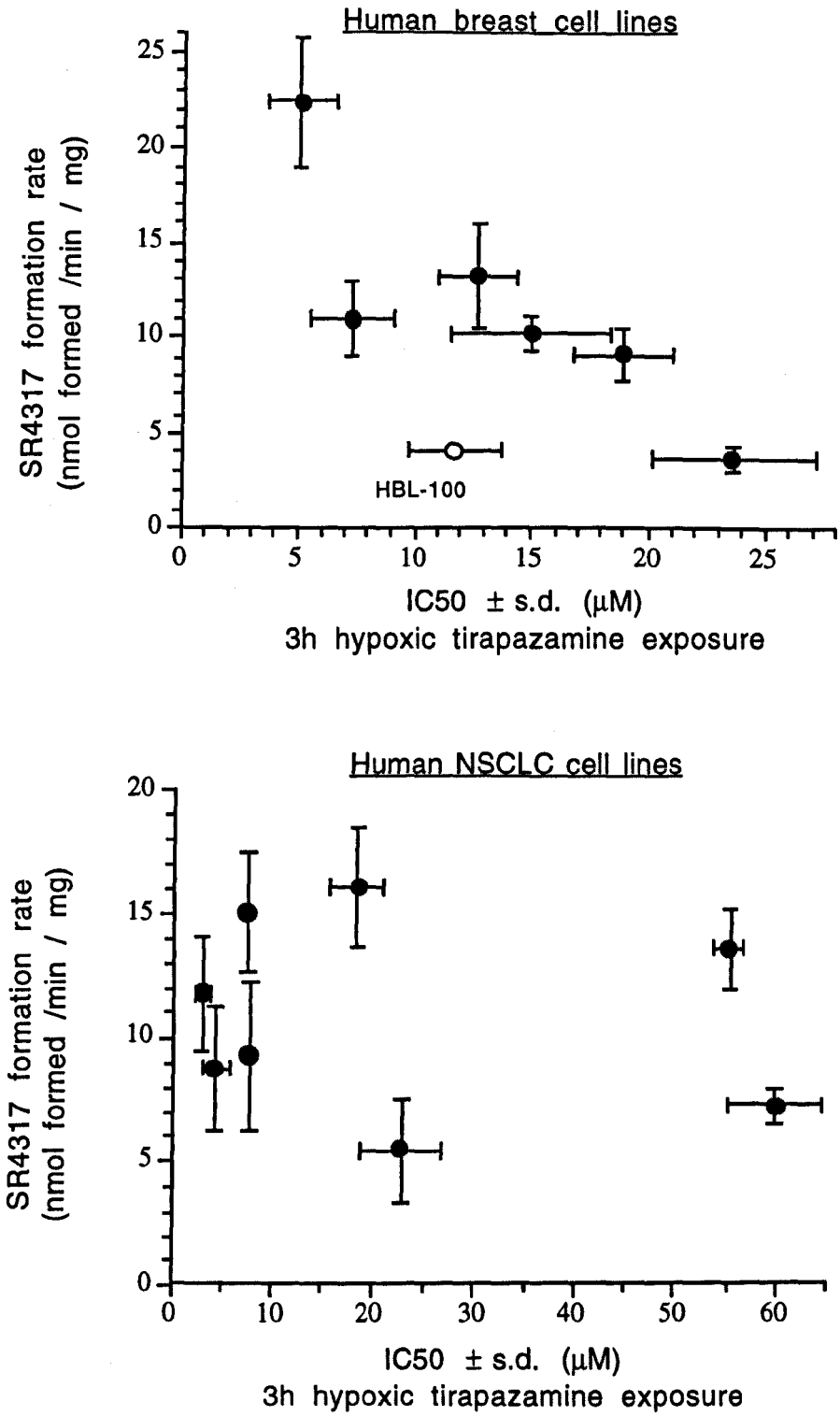


Figure 9 & 10: Human breast & NSCLC cell lines. Relationships between the rate of metabolism of TPZ to its two-electron reduction product, SR 4317, and 3h hypoxic of sensitivity to TPZ.

No correlation between NADH:B_5 reductase and TPZ sensitivity in the cell line panel. A 3-fold range in b_5 reductase activity is seen across the cell line panel. No correlation was found between b_5 reductase activity and hypoxic TPZ sensitivity in either the breast (P=0.81) (figure 11) or lung (P=0.85) (figure 12) cell lines.

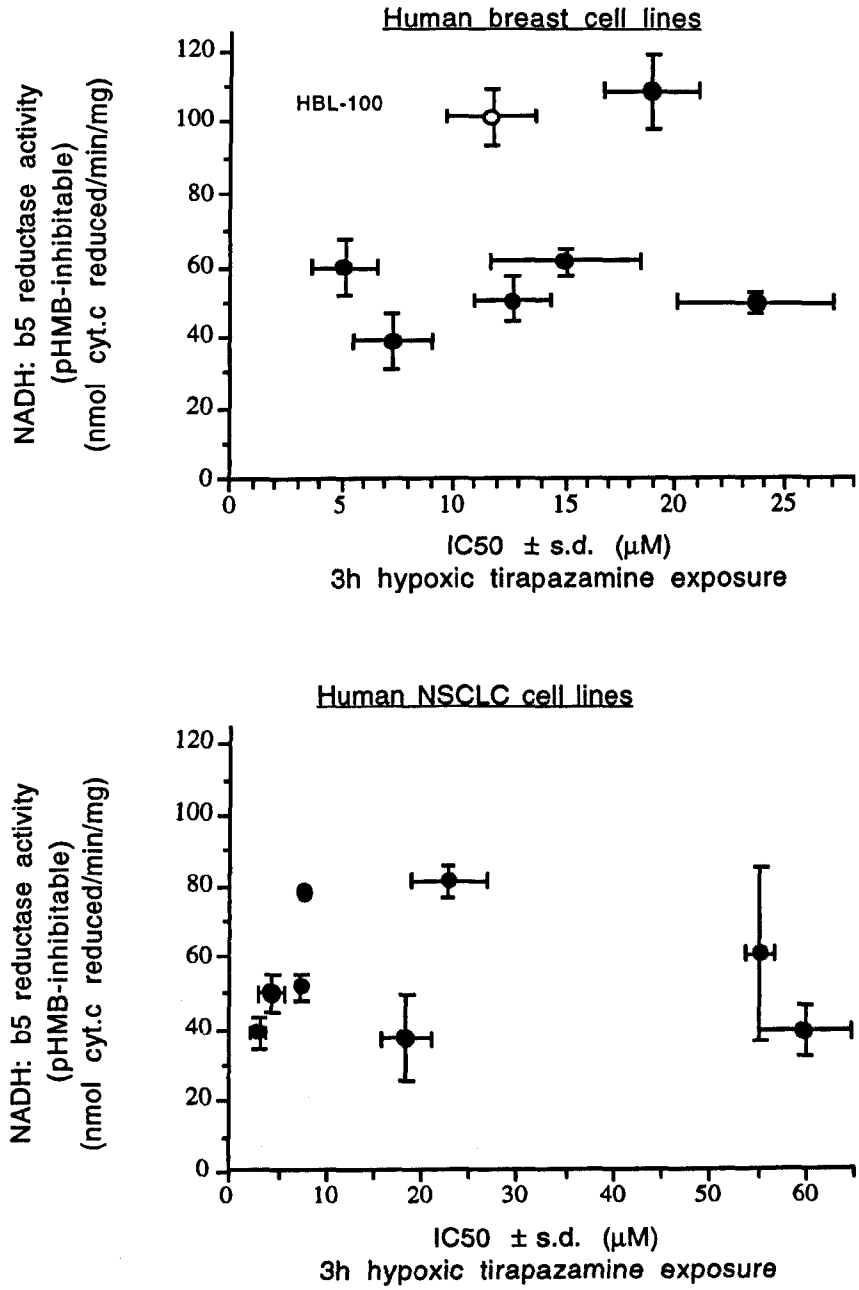
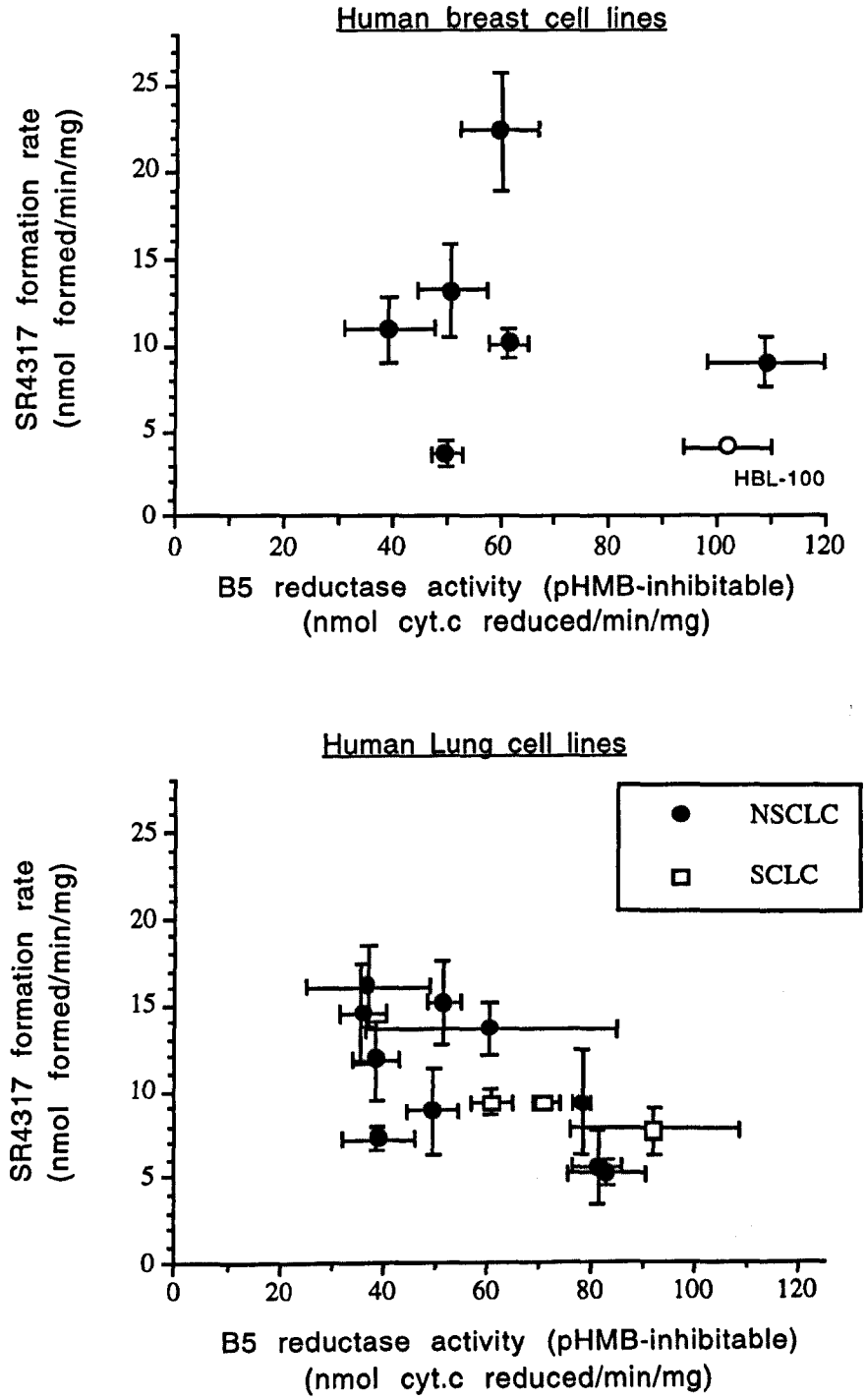


Figure 11 & 12: Human breast & NSCLC cell lines. Relationships between the endogenous b_5 reductase activity and 3h hypoxic sensitivity to TPZ.

In support of this no relationship was seen between b_5 reductase activity and SR 4317 formation activity in either the breast (P=0.88) (figure 13) or lung cell lines (P=0.11) (figure 14).



Figures 13 & 14: Human breast & lung cell lines. Relationships between endogenous b_5 reductase activity and the rate of hypoxic metabolism of TPZ to its two-electron reduction product SR 4317.

No relationship between DT-diaphorase expression and protection from TPZ toxicity in the cell line panel. A 5000-fold range in DT-diaphorase activity was found across the panel of 22 cell lines. Despite these large differences, no correlation was found between DT-diaphorase activity and resistance to chronic TPZ exposure under aerobic conditions (P=0.96) (figure 15) (Patterson *et al.* 1994).

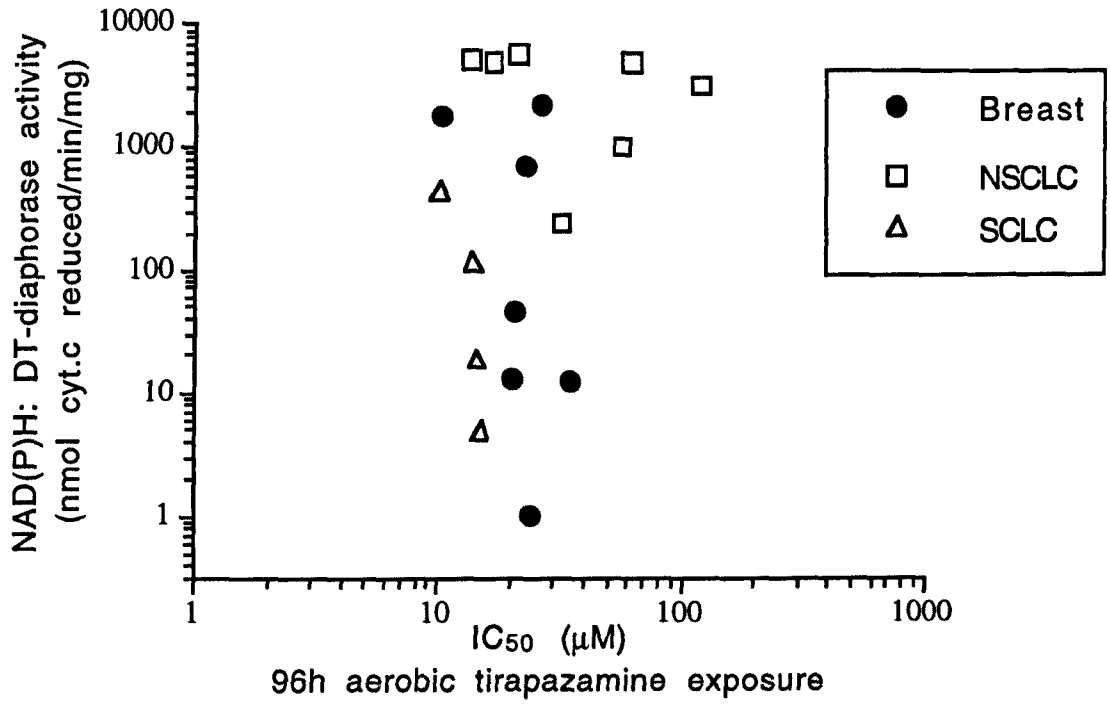


Figure 15: Human breast & NSCLC cell lines. Relationship between the endogenous DT-diaphorase activity and 96h aerobic sensitivity to TPZ.

It has been reported that rat Walker cell DT-diaphorase can efficiently reduce TPZ to its 2-electron reduction product SR4317 (Riley and Workman 1992). Since this reduction is a direct 2-e^- transfer (which operates irrespective of tissue oxygenation status), reduction of TPZ would bypass the postulated cytotoxic nitroxide radical intermediate, providing an effective cellular detoxification mechanism. However it would appear that in contrast to rat, the human DT-diaphorase does not efficiently catalyse this direct two-electron detoxification pathway, and thus is not an important determinant of the aerobic or hypoxic toxicity of TPZ. This is consistent with the observation that when cell lysates were incubated with TPZ and NAD(P)H in air, no SR4317

formation could be detected, despite the presence of very high DT-diaphorase activities in some cell line extracts (eg. ZR-75 and A549). Significant differences in the substrate specificities of rat and human DT-diaphorases have been reported (Walton and Workman, 1990; Beall *et al.*, 1994), and if also true of TPZ, would explain the observations in the panel of cell lines.

Intracellular glutathione levels are not an important determinant of resistance to aerobic TPZ exposure. Glutathione (GSH) is known to be important in the scavenging of superoxide and hydroxyl radical species, and it is the most abundant intracellular source of reduced sulfhydryl groups (Kappus, 1986). GSH can be conjugated to many potentially cytotoxic species through the catalytic activity of cellular glutathione transferases. Although the panel of breast and lung cell lines exhibited a 10-fold range in total intracellular GSH, this did not show any relationship with the 96h aerobic sensitivity to TPZ (P=0.93) (figure 16). This indicates that GSH levels are not the determining factor in the response of these cell lines to chronic aerobic TPZ exposure *in vitro*.

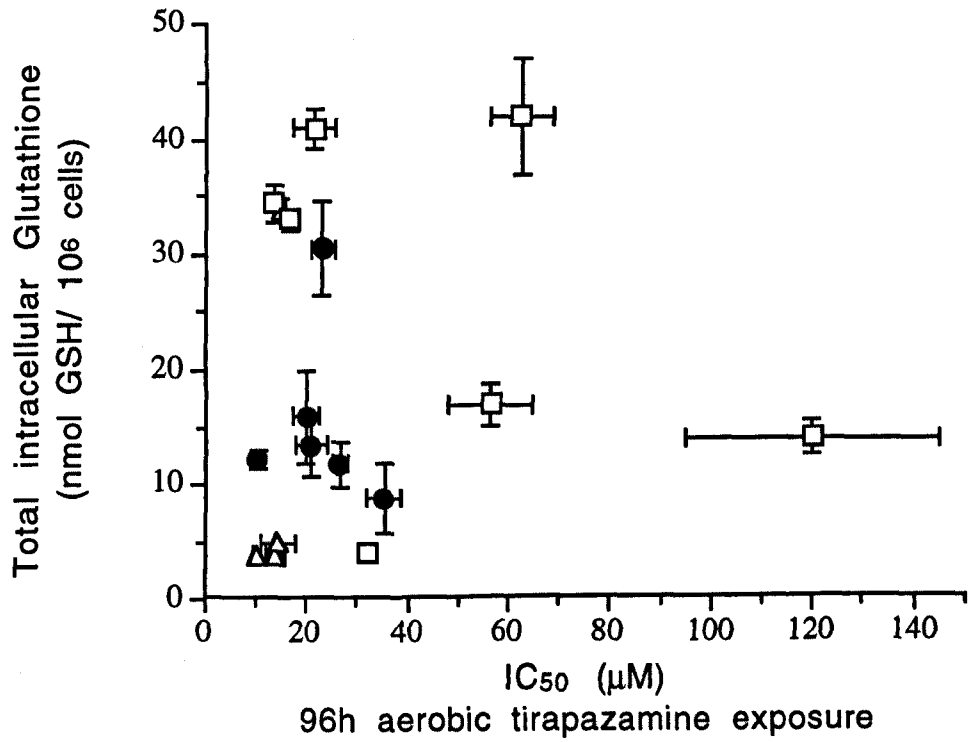


Figure 16: Human breast & NSCLC cell lines. Relationship between the total intracellular glutathione levels and 96h aerobic sensitivity to TPZ.

Western immunoblot of breast cell line SDS extracts. Further evidence to qualify the relationship between P450 reductase expression and catalytic activity was provided by SDS-polyacrylamide gel electrophoresis experiments in which the levels of P450 reductase protein in the breast cell lines was measured using a specific polyclonal antibody for P450 reductase. An equivalent quantity of protein (60μg) from each cell line was loaded onto the gel lanes in rank order of their sensitivity to TPZ (Fig. 17) and a band corresponding to P450 reductase was visualised at 78 Kda. It is apparent that the levels of P450 reductase protein decrease from left to right, i.e. the lowest level of protein occuring in the cell line with the greatest resistance to tirapazamine. When compared with the values determined for catalytic activity in each cell line there is a strong correlation (slope = 2.43 ±0.32; $P<10^{-5}$) (fig. 18).

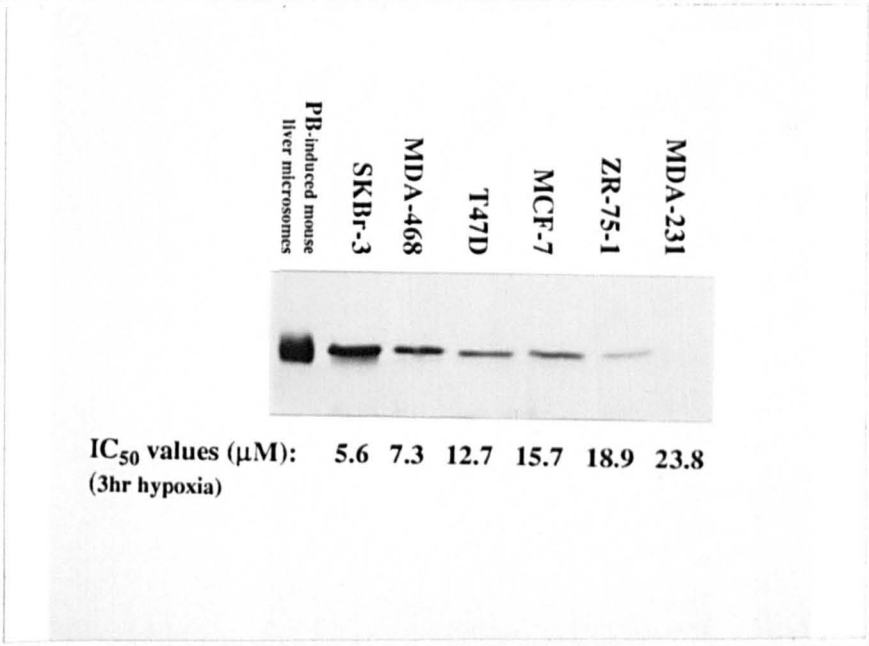


Figure 17: Representative western immunoblot of the relative NADPH: cytochrome P450 reductase expression in the six breast adenocarcinoma cell lines, ranked in order of in vitro sensitivity to TPZ. Gels were loaded with equal total lysate protein for each cell line (67 μg). Microsomes prepared from liver samples of phenobarbital treated mice were used as a positive control (10 μg).

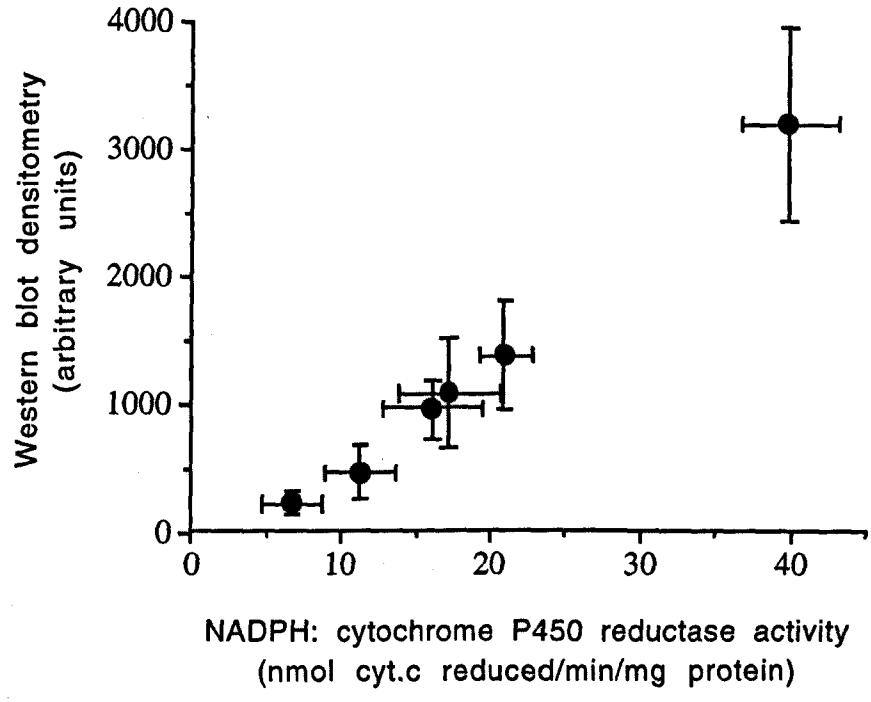


Figure 18: Human breast adenocarcinoma cell lines: Relationship between functional NADPH: cytochrome P450 reductase enzyme activity and total intracellular P450 reductase protein, as determined by densitometric analysis of western immunoblots. Data is mean ± s.d. (n=3).

Compare analysis of the intrinsic sensitivities of the breast and lung cell line panel to a range of unrelated chemotherapeutic agents. This panel of cell lines had previously been adopted to study the mechanisms of multidrug resistance (Houlbrook *et al.* 1994) using a range of commonly used but mechanistically unrelated chemotherapeutic agents (alkylating agents and intercalators). As with TPZ, sensitivities were determined using the MTT proliferation assay (96 h growth) and results were expressed as IC_{50} ± s.d.. This allowed the comparative analysis of the TPZ sensitivities against each of the other chemotherapeutic agent data, for both the breast and lung cell line panels. It was anticipated that this might provide suggestive patterns of sensitivity and resistance amongst the cell lines.

The results were very striking. In the breast cell lines (n=6) the pattern of hypoxic sensitivity to TPZ did not correlate with that seen for any of the diverse chemotherapy agents (table 3). In marked contrast, an identical analysis in the panel of NSCLC cell lines (n=7) revealed a statistically significant correlation (i.e. $P < 0.05$) with all the other agents tested in the lung cell line panel (table 4). Further analysis of the 3h & 96h aerobic TPZ sensitivities of the cell lines, revealed an identical pattern.These data strongly suggests that in the lung cell line panel the

intrinsic sensitivity of each cell line is strongly governed by its capacity to prevent and/or efficiently recognise and repair a diverse set of potentially lethal DNA lesions. Resistance to such a broad spectrum of chemotherapy agents is almost certainly multi-factorial and these characteristics may explain why no relationships were seen between the expression of reductive enzymes necessary to activate TPZ, and the consequential sensitivity to this agent.

Table 3. Human Breast cell lines. Correlations between sensitivity to tirapazamine and other unrelated chemotherapeutic agents.

Agent	Tirapazamine sensitivity		
	96h aerobic	3h aerobic	3h hypoxic
Adriamycin	t_4 = 0.728 (P= 0.51)	t_4 = -1.23 (P= 0.29)	t_4 = 0.524 (P= 0.63)
m-AMSA	t_4 = 0.230 (P= 0.23)	t_4 = -0.47 (P= 0.67)	t_4 = 0.152 (P= 0.89)
Chlorambucil	t_4 = -0.35 (P= 0.74)	t_4 = 0.083 (P= 0.94)	t_4 = 0.311 (P= 0.77)
VP-16	t_4 = 0.825 (P= 0.46)	t_4 = 0.328 (P= 0.76)	t_4 = 0.454 (P= 0.67)
Melphalan	t_4 = 0.868 (P= 0.43)	t_4 = 1.110 (P= 0.33)	t_4 = 1.050 (P= 0.35)
BCNU	t_4 = 0.624 (P= 0.57)	t_4 = 3.35 (P= **0.028**)	t_4 = 1.520 (P= 0.20)
Cis-platin	t_4 = 0.361 (P= 0.74)	t_4 = 1.420 (P= 0.23)	t_4 = 1.660 (P= 0.17)
Mitoxantrone	t_4 = 0.723 (P= 0.76)	t_4 = 0.929 (P= 0.41)	t_4 = 0.245 (P= 0.82)

Table 4: Human NSCLC cell lines. Correlations between sensitivity to tirapazamine and other unrelated chemotherapeutic agents.

Agent	Tirapazamine sensitivity		
	96h aerobic	3h aerobic	3h hypoxic
Adriamycin	t_9 = 2.96 (P= 0.061)	t_5 = 13.0 (P= **0.000048**)	t_5 = 7.60 (P= **0.00063**)
m-AMSA	t_9 = 4.45 (P= **0.0016**)	t_5 = 2.44 (P= 0.059)	t_5 = 3.42 (P= **0.019**)
Chlorambucil	t_9 = 4.01 (P= **0.031**)	t_5 = 8.69 (P= **0.00033**)	t_5 = 13.7 (P= **0.000038**)
VP-16	t_9 = 2.88 (P= **0.018**)	t_5 = 3.50 (P= **0.017**)	t_5 = 4.68 (P= **0.0054**)
Melphalan	t_9 = 2.44 (P= **0.038**)	t_5 = 6.62 (P= **0.0012**)	t_5 = 5.68 (P= **0.0024**)
BCNU	t_9 = 3.34 (P= **0.0087**)	t_5 = 2.62 (P= **0.047**)	t_5 = 3.17 (P= **0.025**)
Cis-platin	t_9 = 2.56 (P= **0.031**)	t_5 = 4.55 (P= **0.0061**)	t_5 = 4.99 (P= **0.0041**)
Mitoxantrone	t_9 = 2.02 (P= **0.034**)	t_5 = 3.01 (P= **0.031**)	t_5 = 3.95 (P= **0.011**)

Tables 3 & 4: Tests for correlation are conducted by fitting a linear functional relationship on a log-log plot, and testing significance of the slope using a student's t-test. The p-values are 2-tailed. **Bold** indicates significance at 5% level or higher (on 9df, $t \geq 2.26$ for 5%, ≥ 3.25 for 1%; on 5df, $t \geq 2.57$ for 5%, ≥ 4.03 for 1%; on 4df, $t \geq 2.94$ for 5% significance).

Profiling of reductive enzymology of *in vivo* xenografts. The comparative activities of the key reductive enzymeswere determined in a panel of breast and lung cell line xenografts.

Table 5: *In vitro & in vivo* determinations of metabolic enzymes in a panel of human cell lines.

Cell lines	Tissue type	NADPH: P450 reductase nmol cyt.c/min/mg		NAD(P)H: DT-diaphorase nmol cyt.c/min/mg		Cytochrome P450 3A BROD O-debenzylation pmol oxidised/min/mg	
		In Vitro	**In Vivo**	**In Vitro**	**In Vivo**	**In Vitro ***	**In Vivo**
SKBr3	Breast	39.8	n/d	1870	n/d	1.89	n/d
MDA-468	Breast	20.9	3.0	13	6.5	1.71	6.1
T47D	Breast	17.2	n/d	46	n/d	n/d	n/d
MCF-7	Breast	18.7	3.5	709	n/d	< 0.1	6.7
ZR-75-1	Breast	11.2	2.5	2290	n/d	< 0.1	6.6
MDA-231	Breast	6.7	3.3	12	23.2	< 0.1	5.5
A549	NSCLC	22.0	4.4	5930	5537	1.38	12.6
CALU-3	NSCLC	21.5	n/d	10	n/d	< 0.1	n/d
SK-MES	NSCLC	19.2	n/d	362	n/d	< 0.1	n/d
NCI-H 322	NSCLC	18.4	n/d	5030	n/d	0.99	n/d
NCI-H 358	NSCLC	17.7	n/d	3290	n/d	< 0.1	n/d
NCI-H 647	NSCLC	9.1	4.5	5140	1960	0.94	12.5
NCI-H 249	SCLC	27.1	7.5	5	n/d	< 0.1	6.4
HT-29	Colon	16.3	6.5	2760	1544	< 0.1	8.3

* Limit of detection for BROD metabolism ≈ 0.1 pmol/min/mg.

(N.B. Human liver BROD activity varies between 4-130 pmol/mg microsomal protein/min). n/d = not determined

Similar *in vivo* and *in vitro* activities were seen within the subset of cell lines with respect to DT-diaphorase and B_5 reductase. Generally the expression of these enzymes was ranked in the same order for both *in vitro* and *in vivo* determinations. In contrast, P450 reductase activity was consistently and markedly down-regulated, such that only a modest 2 -fold difference in activity was found across the xenograft panel (2.5 - 6.4 nmol cyt.c reduced min^{-1} mg^{-1}). This was the opposite pattern to that seen for cytochrome P450 3A activity (BROD oxidation), which was 2- to 10-fold higher than that found in the *in vitro* lysates. *In vitro* and *in vivo* no CYP P450 1A1, 1A2 or 2B activities were detected in any of the cell line or xenograft samples. CYP2C was not assayed due to the lack of an appropriately specific substrate.

2.4 Discussion

Through the application of "enzyme profiling" in a panel of 22 human cell lines it was demonstrated that within the subset of 6 breast adenocarcinoma lines, P450 reductase plays a major role in the metabolic reduction of the TPZ and thus in determining the toxicity of the drug *in vitro*.

The observations in the breast cell line panel that the formation of SR 4317 correlates with P450 reductase activity (figure 7) and that this production of SR 4317 also correlates with the hypoxic toxicity of TPZ (figure 9), implicates the P450 reductase-driven activation of TPZ to its one-electron reduced radical as an important contributory factor in the hypoxic toxicity of this drug. In the application of these observations it is useful to note that the expression of P450 reductase protein (measured by western blotting) correlates well with the activity of P450 reductase (figures 17 & 18), indicating that in each case all the enzyme detected is catalytically active. Thus, enzyme profiling of cells and tissues, together with a measure of tumour hypoxia, may provide a useful screen for predicting the activity of TPZ *in vivo* (Olive *et al.* 1993; Philip *et al.* 1994; Rampling *et al.* 1994; Raleigh *et al.* 1996).

In marked contrast to the breast adenocarcinoma cell lines, no relationships were seen in the panel of 15 NSCLC and SCLC cell lines; with neither the metabolic reduction of TPZ (n=13) nor the sensitivity to the drug (n=7) being apparently dependent upon the cellular complement of P450 reductase. The SR4317 formation data strongly suggests that cellular reductase(s) other than P450 reductase are involved in the metabolism of TPZ. However, since the monitoring of SR4317 formation rate does not differentiate between the relative contributions of either one- or

two-electron reduction pathways, the differences in SR4317 formation will not necessarily relate to toxicity. This is consistent with the lack of correlation between hypoxic IC_{50} values and the velocity of SR 4317 formation, but it also suggests that factor(s) other than drug activation may also contribute to the sensitivity of these cell lines.

This possibility is supported by the statistical analysis of the relative sensitivity of each of the cell lines to a spectrum of unrelated chemotherapeutic agents (table 4), and suggests that intrinsic sensitivity is a distinct characteristic of each cell type irrespective of the chemotherapeutic agent employed. Since most of the agents tested do not depend upon specific cellular metabolism to manifest their cytotoxic effects, and are known to have distinct modes of action, the correlations found between TPZ and these unrelated compounds suggests that the diverse histological subtypes represented in the lung cell line panel dominate the pattern of response to drug induced growth inhibition. Indeed, Houlbrook *et al.* (1994) demonstrated that this lung cell line panel showed significant inter-drug correlations, with the calculation of Kendall's coefficient of concordance giving a value of 0.69 (P= 0.0001), indicating that if a cell line is resistant to one drug it is likely to be resistant to many others. This is arguably consistent with the patterns of response seen clinically; where SCLC is usually highly responsive to first line chemotherapy, while NSCLC is generally multidrug resistant at presentation and consequently refractory to treatment.

However, it is far from clear which other reductase(s) are involved in the metabolism of TPZ in these cell lines *in vitro*. The majority of published studies have focused upon microsomal metabolism of TPZ, since by far the greatest proportion of enzyme activity resides in this subcellular fraction. Walton and Workman (1990) have shown that anaerobic TPZ metabolism by mouse liver microsomes was some 40-fold higher than equivalent cytosolic-dependent reduction, with this microsomal reduction displaying a strong preference for NADPH over NADH as a source of reducing equivalents. Cytosolic reduction in contrast was found to be equally dependent on NADH and NADPH. Although a considerable number of publications have implicated or defined the contributions of a number of reductive enzymes in TPZ metabolism, for the majority, the relevence to TPZ cytotoxicity is not particularly clear. A critical analysis of the published literature serves to illustrate this uncertainty.

2.4.1 Microsomal metabolism

2.4.1.1 NADPH: cytochrome P450 reductase.

EPR spectroscopic studies (Lloyd *et al.*, 1991) utilising rat liver microsomes identified NADPH: cytochrome c (P450) reductase (EC 1.6.2.4, P450 reductase) as the major hepatic microsomal enzyme responsible for the reduction of TPZ to a one-electron nitroxide radical intermediate, which in its protonated form, is thought to be the cytotoxic species (Costa *et al.*, 1989; Wang *et al.*, 1992; Brown, 1993). In agreement with Lloyd *et al.* (1991), incubation of TPZ with purified rat liver P450 reductase under nitrogen has been shown to lead to the production of strand breaks in co-incubated plasmid DNA (Fitzsimmons *et al.*, 1994). Other studies have shown that TPZ is reductively metabolised to SR4317 by purified rat liver P450 reductase (Cahill and White, 1990; Fitzsimmons *et al.*, 1994). The rate of SR4317 formation by in vitro cell lysates has been linked to cytotoxicity under anaerobic conditions (Biedermann *et al.*, 1988; Costa *et al.*, 1989; Wang *et al.*, 1993). Further, Silva and O'Brien (1993) demonstrated that freshly harvested rat hepatocytes were highly sensitive to TPZ under hypoxia, but if preincubated with the flavoprotein (i.e. P450 reductase) inhibitor, diphenylene iodonium (DPI^+) (Doussiere and Vignais, 1992), both cytotoxicity and TPZ metabolism were prevented. In agreement, in this chapter it was showed that the formation velocity of SR 4317 correlated with P450 reductase activity, and that the rate of SR 4317 formation in turn correlated with the hypoxic toxicity of TPZ. These observations implicated the P450 reductase-driven activation of TPZ as a major contributory factor in the hypoxic toxicity of this drug in vitro.

However contradictary evidence has recently been published, questioning the role of P450 reductase in the bioactivation of TPZ in vitro (Elwell *et al.*, 1997). Elwell and colleagues adapted the A549 human lung adenocarcinoma cell line to chronic aerobic TPZ exposure in incremental steps over a period of six months. In the cell lines adapted to 25-100 μM TPZ, P450 reductase activity was reduced to only 1-3% of the parental value, yet total cellular bioreductive capacity, determined as production of SR 4317 under hypoxic conditions, was only reduced 2-fold. In turn, only a 1.5-fold decrease in hypoxic TPZ sensitivity was seen. This contrasted with the 1.8 to 9.2 -fold range in increased resistance to cell killing by TPZ under aerobic conditions. An alternative example of P450 reductase down-regulation following chronic exposure to a reductively-activated cytotoxin (mitomycin C) has been reported in a Chinese hamster ovary cell line, MMC^R (Hoban *et al.*, 1990), but in this case cross-resistance to TPZ was found under <u>both</u> aerobic and hypoxic conditions (Keohane *et al.*, 1990).

Interestingly, these incremental adaptions in aerobic resistance observed by Elwell *et al.* (1997) were mirrored by similar magnitudes of elevation in mitochondrial manganese superoxide dismutase activity (MnSOD), with the most resistant population (100 μM^R) being 9.2-fold more resistant to TPZ under normoxia, and having a 9.4-fold elevation in MnSOD activity. The data thus provided good statistical evidence to suggest a role for MnSOD in the acquisition of aerobic TPZ tolerance ($r = 0.966$), and agrees with Silva and O'Brien (1993) in implicating the mitochondrial compartment as playing an important role in aerobic detoxification of TPZ metabolites. Multiple changes in cellular biochemistry often occur following chronic exposure of a mixed population of cells to an escalating negative selection (in this case, increasing doses of TPZ). The changes in P450 reductase, MnSOD and glutathione reductase are only three changes reported by Elwell et al. (1997) that appear to relate to aerobic TPZ tolerence. Unexpectedly, it was also discovered that DT-diaphorase was markedly down-regulated in the adapted populations -- despite its proposed obligate two-electron detoxifying role in TPZ metabolism (Walton and Workman, 1990). In the course of any such long term adaptions, other metabolic enzymes(s) might also become dis-regulated, to compensate for loss of function and so maintain metabolic viability. If true, one would anticipate that other putative one-electron reductase(s) may be up-regulated, which would not significantly metabolise TPZ under aerobic conditions, but could contribute to the hypoxic metabolism of TPZ in the adapted cells. Indeed, Wang and colleagues (1993) identified 30-40% of the reductive TPZ metabolism as belonging to as yet unidentified enzymes. If some other reductase(s) could compensate for P450 reductase in its critical NADPH-dependent flavoprotein functions, including the reduction of TPZ, the contradiction might be resolved. Perhaps approaches such as differential display PCR could aid in the identification of the other putative reductase(s) that contribute to TPZ metabolism.

2.4.1.2 Cytochrome P450 (CYP450).

In vitro studies with mouse liver microsomes and human tumour lysates have demonstrated that a large proportion (58-86%) of all TPZ reductive metabolism is catalysed by CYP450 (Walton and Workman, 1990; Walton *et al.*, 1992; Riley and Workman, 1992; Riley *et al.*, 1993; Wang *et al.*, 1993). While the oxidative metabolism of organic substrates by CYP450 isozymes is well recognised, far fewer studies have explored their role as a reducing catalyst. For a better understanding of the relationship of CYP450 reductive activity to the metabolism of TPZ, it is pertinent to review the role and functions of CYP450 in the reductive metabolism of other model substrates.

The CYP450 hemoproteins exhibit four different types of activity. They can act as a monooxygenases, reductases, peroxidases and oxidases. In its capacity as a reductase, the metabolism of carbon tetrachloride and halothane has been studied most extensively (Johansson and Ingelman-Sundberg, 1985; de Groot and Noll, 1986; Reinke *et al.*, 1988; Van Dyke *et al.*, 1988). Following the interaction and binding of a polyhalogenated alkane substrate with the CYP450, the first one-electron transfer occurs, usually provided by NADPH via the flavoprotein P450 reductase. The CYP450 heme Fe(III) is reduced to Fe(II) while P450 reductase is concurrently oxidised to the stable semiquinone radical. After the release of a halide ion, a haloalkane radical is formed which can either leave the active site or undergo a second one-electron reduction. Thus CYP450 isozymes are essentially two-electron reductases, although they receive their electrons from P450 reductase as two sequential one-electron transfers (and in some cases from cytochrome b_5 via cytochrome b_5 reductase) (Peterson and Prough, 1986). In the absence of competition with oxygen, substrates can dissociate after the transfer of only a single electron, or the reduced CYP450 is able to donate its one-electron to an alternate electron acceptor. Therefore in some cases, the availability of a second electron in the catalytic cycle of CYP450 via P450 reductase or cytochrome b_5 may influence the extent of radical generation (Pompon and Coon, 1984; Peterson and Prough, 1986; Guengerich, 1991).

Because of the low reduction potential of CYP450, a variety of compounds are reducible including; aliphatic N-oxides, azo-dyes and epoxides, which undergo apparent stepwise 2-electron reductions (Levine and Raza, 1988; Koop, 1992). Other substrates are reductively activated by CYP450 in a similar way to the polyhalogenated alkanes (Levine and Raza, 1988; Docampo and Moreno, 1990; de Groot and Sies, 1989; de Groot and Noll, 1986; van de Straat *et al.*, 1987; Vromans *et al.*, 1990; Goeptar *et al.*, 1993). Direct electron spin resonance techniques have been employed to show that gentian violet undergoes a CYP450 dependent one-electron reduction, with formation of the free radical species being strongly inhibited by O_2, carbon monoxide (CO) and metyrapone (Harrelson and Mason, 1982; Docampo and Moreno, 1990). Comparable inhibitable properties have been observed for 3,3'-dichlorobenzidine (Iba and Lang, 1988) and benidazole (Masana *et al.*, 1984). Similarly, the one-electron reduction of the model quinone 2,3,5,6-tetramethylbenzoquinone, or the anticancer agents mitomycin C and doxorubicin to semiquinone free radicals, is strongly suppressed by the well known type I inhibitor of cytochrome P450, SKF 525A (Vromans *et al.*, 1990; Goeptar *et al.*, 1992; 1993). Thus, as a consequence of the potential contributions from either or both 1 and 2-electron transfers arising from the CYP450-dependent reductive metabolism, the possible outcomes are not necessarily predictable; being dependent not only upon substrate specificities and the degree of hypoxia, but also upon the composition of the experimental CYP450 system in both

microsomal and intact cell systems. Yet, while the above examples serve to illustrate the metabolic flexibility of CYP450 reduction, caution is indicated in the interpretation of the mechanisms, since direct parallels between nitrogen, oxygen and carbon centred reductions by CYP450 should not be made.

The contribution of the CYP450 isozymes in the hypoxia-dependent cytotoxicity of TPZ is uncertain, particularly since cultured cells widely used to study drug activation and toxicty, rapidly lose their ability to express the P450 gene family (Paine, 1990). Published studies on the metabolism of TPZ using mouse liver microsomes (Walton and Workman, 1990; Walton *et al.*, 1992; Riley and Workman, 1992; Riley *et al.*, 1993), tumour cell lysates (Wang *et al.*, 1993) and purified enzymes (Walton *et al.*, 1989; Walton and Workman, 1990), have shown that both CYP450 isozymes and P450 reductase contribute to the overall two-electron reduction of TPZ to its non-toxic mono-N-oxide, SR4317, while studies with rat liver microsomes attribute all reductive activity exclusively to P450 reductase (Cahill and White, 1990; Lloyd *et al.*, 1991).

Dexamethasone induced and uninduced mouse liver microsomes, in combination with both chemical- (CO, metyrapone, SKF-525A, tolbutamide, p-nitrophenol) and immuno-inhibitors, have been employed to identify the metabolic contribution of the cyp450 isozymes (Riley *et al.* 1993). Riley and colleagues confirmed earlier observations (Walton and Workman 1990; Walton *et al.*, 1992) that these cyp450 isozymes contributed at least 70% of the total SR4317 formation, with P450 reductase apparently accounting for the remaining 30%. Detailed CYP450-specific inhibitor studies resulted in the identification of the cyp450 2B (53%) and 2C (26%) families as contributing significantly to SR4317 formation under hypoxic conditions. However, the potential contributions of the one and two electron reduction pathways were not discussed. Some corroborative evidence in human tissues (Lewis *et al.*, 1996) has also been demonstrated. Immuno-profiling of CYP450 isozymes in a panel of human liver microsomes (western blotting) and the relationship to hypoxia-dependent SR4317 formation rates has proved significant for CYP450 2B6 ($r = 0.883$, $P = 0.002$) but not CYP450 2C8 or 2C9. Lewis *et al.* also showed that cell lysates from a human lymphoblastoid cell line (Crespi, 1991; Crespi *et al.*, 1993) transfected with a mammalian expression vector to stably express the CYP450 2B6, demonstrated a 1.9-fold increase in TPZ metabolism relative to control lysates, as determined by the measurement of SR4317 formation velocity. However, the relative sensitivities to in vitro TPZ exposure under oxic and hypoxic conditions of the parental and the CYP450 2B6-expressing cell line have not been published.

Wang and colleagues (1993) reported that CYP450 apparently contributed > 60% of the metabolism of TPZ under hypoxic conditions in a mouse (SCCVII) and a human sarcoma (HT1080) cell line lysates; as implied by the observation that CO inhibited the NADPH-dependent reduction of TPZ. Such a result is surprising in view of the low levels of CYP450 activity in most *in vitro* cultured cell lines (Paine, 1990). Indeed, utilising cell lysates from the *in vitro* HT1080 cell line, to catalyse the O-dealkylation of specific alkyloxyresorufin analogues (Burke *et al.*, 1975; 1994), we were unable to detect CYP450 1A1, 1A2, 2B or 3A activities *in vitro* (Table 1). Experiments in our laboratory using *in vitro* HT1080 lysates have shown that metyrapone did not inhibit the NADPH-dependent reduction of TPZ to SR 4317, while the flavoprotein inhibitor DPI^{+} effectively reduced SR 4317 formation velocity by over 90%. These observations raise the intriguing possibility that *in vitro*, TPZ is reductively metabolised by a non-CYP450 hemoprotein that is not down-regulated by long-term culturing, but is (by its heme-containing nature) inhibited by CO. In support of this possibility, it has been shown that the tertiary aliphatic di-N-oxide AQ4N, a bioreductive antitumour agent, is reduced by recombinant nitric oxide synthase (EC.1.14.13.39) (LH Patterson & SM Raleigh, unpublished observations), P450 reductase and free haem (Raleigh *et al.*, 1995). Both reactions are dependent on NADPH and inhibited by CO.

As discussed earlier, Lloyd *et al.* (1991) concluded that all of the rat hepatic microsomal TPZ reduction could be attributed to P450 reductase, finding no observable contribution from the cyp450 isozymes, with neither CO nor metyrapone having any impact upon the EPR signal. Cahill and White (1990) also demonstrated that SR4317 formation was not influenced by CO in rat hepatocytes, and again suggested a dominant role for P450 reductase. It is unlikely that the disagreement over the involvement of the cyp450 system has arisen through a technical problem with the carbon monoxide inhibition, since metyrapone-based inhibition produces the same observed contradiction. One possibility is that these observations could reflect differences between mouse and human on the one hand and rat on the other. Although tertiary amine N-oxides have been shown to undergo reduction by cyp450 in rat liver microsomes, formation velocities and cofactor requirements imply a distinctly more complex mechanism of NADPH-dependent metabolism (Bickel, 1969; Sugiura *et al.*, 1974, 1976, 1977; Kato *et al.*, 1978). Of those studies which have implied a dominant role for murine cyp450 and human CYP450 (Walton and Workman, 1990; Walton *et al.*, 1992; Riley *et al.*, 1993; Wang *et al.*, 1993), all have monitored the formation velocity of the two-electron reduction product SR4317 by HPLC. Since the measurement of SR4317 production under hypoxic conditions is only a surrogate measure for the formation of the nitroxide radical, it makes no discrimination between the enzyme-mediated conversion of TPZ to its one-electron reduced product (with its subsequent chemical conversion to the mono-N-oxide, SR4317), and any direct two-electron reduction events which would

bypass the cytotoxic radical intermediate. Lloyd and colleagues directly monitored the steady-state generation of the nitroxide radical by EPR spectroscopy, unambiguously ruling out a role for the rat cyp450 system in the reductive activation of TPZ. Neglecting the possible differences in the orthology of rodent cyp450, it might be argued that the results of rat *versus* mouse liver microsomes are consistent if one postulates of that the murine P450 isozyme-dependent reduction of TPZ is not proceeding *via* the nitroxide radical, but instead represents a potent two-electron detoxification pathway (i.e. 58-86% of total metabolism). TPZ could interact with the ferrohaemoprotein form of CYP450, generated following NADPH:cytochrome P450 reductase mediated reduction. The interaction with TPZ would be followed by a direct two-electron oxidative cleavage of the [Fe-O-N] complex to generate Fe^{IV}=O and mono-N-oxide, and is effectively a "TPZ-detoxifying" oxygen atom transfer process. A further one-electron transfer by P450 reductase would generate the ferric form of CYP450 ready for the next catalytic cycle. A similar mechanism has been proposed for aliphatic N-oxide reduction (Suguira, 1976; 1977). Only EPR spectroscopic analysis of the effects of CYP450 inhibitors on anoxia-dependent nitroxide radical formation by mouse and human microsomes or reconstituted systems will resolve this uncertainty.

Other factors must question the apparent dominance of CYP450 in the reductive metabolism of TPZ. The reduction rate of TPZ to SR4317 by both uninduced and induced mouse liver is extremely rapid, having reported values of 182 to 372 nmol/min/mg microsomal protein (Riley et al., 1993). Similar values have been reported for human liver microsomes, with values ranging from 88 to 197 nmol/min/mg microsomal protein (Lewis *et al.*, 1996). The spectrally determined cyp450 content of mouse liver microsomes used by Riley et al. was 0.65 to 1.83 nmol cytochrome P450 / mg microsomal protein, for control and dexamethasone treated animals respectively. Since most CYP450 reactions are relatively slow, with rates of ≈ 1 nmol of product formed / nmol of cytochrome P450 / min being typical for many substrates (Guengerich, 1991), this apparent cyp450-dependent metabolism is proceeding 200 - 280 times more rapidly than might be predicted. Of note, Lloyd and colleagues (1991) pointed out that their V_{max} (900 nmol/min/mg protein) and K_m (1.4mM) values determined for futile cycling of TPZ were in exellent agreement with the values determined for the anaerobic metabolism of TPZ by mouse liver microsomes (Walton and Workman, 1990). It was concluded that such a large cyp450-dependent V_{max} value was hard to understand, although these catalytic rates were typical of P450 reductase (Fitzsimmons *et al.*, 1994).

Consistent with the literature on rat liver metabolism, Silva and O'Brien (1993) found that the inhibition of cyp450 by metyrapone or SKF-525A in intact rat hepatocytes, did not affect the

reduction of TPZ under hypoxic conditions (< 0.1% O_2). Subsequently, Khan and O'Brien (1995) extended the work in this freshly harvested rat hepatocyte model to demonstrate that under 1% O_2, metyrapone and SKF-525A, (as well as cimetidine and erythromycin), could not protect against TPZ cytotoxicty as defined by trypan blue exclusion. However they found that the cyp450 2E1-specific inhibitors (substrates), ethanol, isopropanol, phenylimidazole and isoniazid afforded significant cytoprotection, and suggested that TPZ was activated by rat cyp450 2E1 as a result of a redox cycling-mediated oxygen activation. The data extensively implicates reactive oxygen species (ROS) in the TPZ dependent cytotoxicity at 1% O_2, since glutathione-depleted as well as catalase or glutathione reductase-inactivated hepatocytes were more sensitive to cell killing by TPZ. The disulphide reductant DTT (5mM) also prevented TPZ cytotoxicity, although at this concentration it is an effective inhibitor of cyp450 2E1 activity (Yoo *et al.*, 1987).

In support of the dominant involvement of ROS, polyphenolic antioxidants quercetin, purpurogallin and caffeic acid, which scavange superoxide radicals, effectively prevented both cytotoxicity and GSH oxidation, although the lack of protection afforded by phenolic antioxidants implied that lipid peroxidation was not an important determinant of the TPZ cytotoxic mechanism. Further, the radical trap and superoxide dismutase mimic, Tempol, or the ferric chelator desferrioxamine (DFX), prevented hepatocyte toxicity, perhaps implicating the generation of hydroxyl radicals by the Haber-Weiss reaction. The role of the nitroxide radical itself was not discussed in detail, although the authors concluded that " *under 1% O_2, reduced cytochrome 2E1 is more effective than cytochrome P450 reductase at carrying out a one-electron bioreduction of tirapazamine to the tirapazamine radical which redox cycles and forms cytotoxic reactive oxygen species* ". However, although Khan and O'Brien (1995) briefly state that "*tirapazamine was just as cytotoxic to hepatocytes under 1% oxygen as was previously observed under nitrogen*", no evidence is provided to demonstrate the suggested one-electron reductive role of cyp450 2E1 under anoxia.

Several difficulties complicate the interpretation of these data. Firstly, a specific inhibitor of cyp450 2E1 was not employed, it is not possible to unequivocally establish the participation of cyp450 2E1 in TPZ metabolism. The use of reversible inhibitors is less effective, and in most cases the chemicals represent alternative substrates and/or ligands for other P450s as well. As the inhibition depends on the binding constant to cyp450 2E1 and to other forms of cyp450, some selectivity may be observed, but it is often difficult to interpret. Several compounds have been suggested to be specific mechanism-based inhibitors of cyp450 2E1 (Koop, 1990; Gannett *et al.*, 1990). Secondly, the inclusion of 0.2% (v/v) DMSO (28 mM) in the final incubations complicates the interpretation, since DMSO is an effective competitive inhibitor of cyp450 2E1, with a K_i =

0.39 mM (Yoo et al., 1987). DMSO is also a competitive inhibitor of imidazole-induced microsomal activity (Kaul and Novak, 1984). Thirdly, Riley *et al.* (1993) ruled out a role for murine cyp450 2E1-dependent TPZ reduction through the use of an inhibitory monoclonal antibody (mAb 1-91-3), although 11% inhibition was seen in induced microsomes with the cyp450 2E1 substrate p-nitrophenol. However, DMSO was present in the final incubation questioning the apparent lack of effect. In tentative agreement, Lewis *et al.* (1996) failed to find any correlation between CYP450 2E1 expression and TPZ reduction in a panel of human liver microsomes ($r = -0.40$, $P = 0.286$). Fourthly, the lack of cytoprotection by metyrapone and SKF-525A argues against the involvement of a one-electron reduction, since free radical formation of some other substrates by cyp450 has been shown to be inhibited by these agents (Harrelson and Mason, 1982; Masana *et al.*,1984; Iba and Lang, 1988; Vromans *et al.*, 1990; Goeptar *et al.*, 1992, 1993). Finally the use of 1% O_2 in the cytotoxicty experiments markedly complicates interpretation, since molecular oxygen itself is an exellent substrate for reductive reactions.

This oxidase activity of CYP450 2E1 is as important, if not more so, than the reduction of model substrates like CCl_4. This isozyme is constitutively in its high-spin state (Koop *et al.*, 1982) and can readily accept electrons from P450 reductase in the absence of substrate. This not only confers the ability to efficiently reduce substrates such as CCl_4, but it makes the enzyme especially effective in utilising dioxygen as an electron acceptor (Persson *et al.*, 1990; Koop, 1992). Consequently, purified CYP450 2E1 exhibits a higher rate of oxygen consumption and oxidase activity than other CYP450 forms (Gorsky *et al.*, 1984). Such enhanced oxidase activity would operate efficiently at 1% O_2 (10.5 μM) (Persson *et al.*, 1990) and would result in the increased production of both superoxide and hydrogen peroxide. This oxygen-dependent activity in the presence of chelated iron can produce reactive hydroxyl radicals (Dicker and Cederbaum, 1987; Feierman *et al.*, 1985), and is exemplified by the use of reconstituted systems in which cyp450 2E1 displays a unique ability to potentiate iron-catalysed Fenton chemistry. Of note, both DFX and Tiron have been shown to confer protection against TPZ aerobic, but not hypoxic cytotoxicity (Herscher *et al.*, 1994). These oxidase-generated ROS would be indistinguishible from the ROS arrising from the postulated "*redox cycling-mediated oxygen activation*" if one speculated that TPZ could enhance the level of cyp450 oxidase activity or could itself donate mono-oxygen. Indeed some evidence exists for substrate-induced activation of oxygen through the uncoupling of cyp450 (van de Stratt *et al.*, 1987). Two quinone imine substrates studied by van de Stratt *et al.* were themselves unable to redox cycle with oxygen, but in aerobic microsomal incubations they nevertheless produced marked increase in the formation of H_2O_2 and hydroxyl free radicals, thus appearing to stimulate the oxidase activity of cyp450.

Studies with liver microsomes and some purified cyp450s *in vitro*, indicate that a large fraction of the reducing equivalents of NADPH is used nonproductively, with the extent of uncoupling being dependent on the cyp450 form (Gorsky *et al.*, 1984; Guengerich, 1991). However the *in vivo* significance of these nonproductive reactions is still uncertain. Thus the conjecture that the oxidase function of cyp450 2E1 is involved in the hepatocyte cytotoxicity of TPZ observed by Khan and O'Brien is not deducible from the literature, with ambiguous evidence either way. However a comparative analysis of the inhibitors that prevented TPZ-dependent cytotoxicity *in vitro* (Khan and O'Brien, 1995), against independent studies (Persson *et al.*, 1990; Bondy and Naderi, 1994) that evaluated their relative capacity to moderate cyp450 2E1 oxidase activity in a purified rat microsome system would tentatively suggest no such relationship. While ethanol and isoniazid were both capable of cytoprotection, only isoniazid produces significant inhibitory effects (70%) on cyp450 2E1 oxidase function in a reconstituted system. However, since the studies were conducted at different pO_2, and often differing inhibitor concentrations, it is difficult to draw any firm conclusions. Unfortunately no corroborative evidence regarding CYP450 2E1 mediated metabolism has been published. It would be of significant interest to expand these experiments in isoniazid-induced hepatocytes (Koop, 1990; Koop and Tierney, 1990), in conjunction with mechanism-based CYP450 2E1 inhibitors and in the absence of both DMSO and O_2.

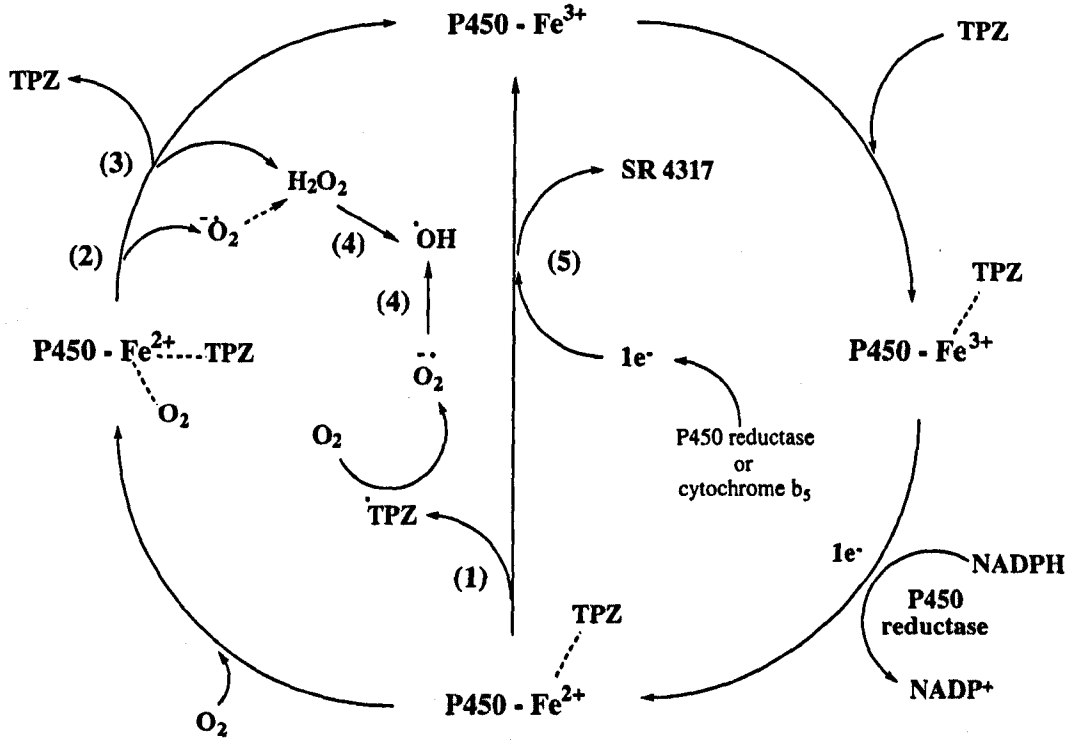


Figure 19: Possible mechanisms of action of cytochrome P450 (CYP450) in the reduction of tirapazamine (TPZ) in the presence of molecular oxygen. (1) The single-electron reduction of TPZ by CYP450 with subsequent futile cycling of the nitroxide radical intermediate. (2) Oxidase activity of CYP450. (3) Dioxygen may compete for the transfer of electrons to TPZ, giving rise to an uncoupling of the CYP450 reaction cycle, with concomitant formation of reactive oxygen species (ROS). (4) ROS arising from TPZ-mediated activation of molecular oxygen or from a TPZ-dependent CYP450 uncoupling mechanism. (5) CYP450 may catalyse the concerted two-electron reduction of TPZ, particularly if a second electron is readily available.

There is good evidence that haemoproteins including CYP450 and haemoglobin can, in the absence of dioxygen, two-electron reduce N-oxides to their corresponding amines (Bickel, 1969; Powis & McGraw, 1980, Burka *et al.*, 1985). Indeed, TPZ has been shown to be reduced significantly to its mono-N-oxide (SR 4317) and zero-N-oxide (SR 4330) in hypoxic incubations containing mouse whole blood, but not plasma (Walton & Workman, 1993). Mechanistically N-oxides can be reduced by haem centres by a process requiring haem-N-oxide co-ordination to generate an [Fe-O-N] complex. A number of N-oxides have been shown to undergo type II spectral interactions with CYP450 in the absence of oxygen (Lindeke & Paulsen-Sorman, 1988). Such spectra are considered to be a result of direct coordination of the N-oxide (acting as a lone pair electron donor) with haem iron (Fe) of CYP450 to produce the [Fe-O-N] complex.

Taken as a whole, no publication has yet to unambiguously assign a role for the CYP450 system in the one-electron reductive bioactivation of TPZ, despite numerous demonstrations of the dominant role of murine cyp450 isoforms in mono-N-oxide formation. Thus, while evidence suggests human CYP450 may have some role in the overall metabolism of TPZ, it still remains unclear to what extent and in what context the CYP450 isozymes are important in the reductive activation and the subsequent cytotoxicity of TPZ.

2.4.1.3 NADH: ferricytochrome b_5 reductase.

No reports have been published to suggest a role for other recognised microsomal reductases such as NADH-cytochrome b_5 reductase and cytochrome b_5 in the metabolism of TPZ. Limited studies in our laboratory using the inhibitor p-hydroxymecuribenzoate (Barham *et al.*, 1996), have suggested a role for NADH-b_5 reductase in TPZ metabolism, for the panel of 15 human lung cell

lines (Chinje & Patterson, unpublished). Evidence for the involvement of cytochrome b_5 reductase in the reductive activation of a fused pyrazine mono-N-oxide (RB90740) has also been reported (Barham and Stratford, 1996). Further, the potential involvement of NADH-b_5 reductase and cytochrome b_5 in cytochrome P450-dependent one- or two-electron reductive metabolism of TPZ is undefined.

2.4.2 Mitochondrial metabolism

P450 reductase, CYP450 and DT-diaphorase are all present in the mitochondrial matrix, while b_5 reductase is found in the outer membrane of mammalian mitochondria (Prebble, 1981; Lind & Hojeberg, 1991; Waterman, 1982). Mitochondrial CYP450s receive their electrons from NADPH via the flavoprotein adrenodoxin reductase and the iron-sulphur protein adrenodoxin (Waterman, 1982). Spanswick *et al.* (1996) have recently identified a novel NADPH-dependent one-electron reductase capable of metabolising mitomycin C under anoxic conditions. Of the defined and uncharacterised mitochondrial reductases, limited evidence is available regarding their relevance to TPZ metabolism. Perhaps of significance, mitochondrial DNA has been identified as an important therapeutic target for mitomycin C-mediated damage (Pritsos *et al.*, 1997), raising the possibility that the activity of these mitochondrial reductases might also be of importance in localised TPZ activation.

Silva and O'Brien (1993) provided evidence that metabolites of TPZ can act as substrates for the mitochondrial respiratory chain. Incubation of TPZ (but not SR 4317 or SR 4330) with respiring rat liver mitochondria, induced NADH-dependent state 3 and 4 mitochondrial respiration, which was completely inhibited by cyanide and antimycin A but not rotenone. This implied a potential detoxification mechanism, in which the nitroxide radical could redox cycle via the mitochondrial electron transport chain without generating oxidative stress through oxygen activation. Cytotoxicity data supported this theory, since addition of a nontoxic concentration of KCN markedly narrowed the differential in TPZ sensitivity under oxic and hypoxic conditions. Interestingly, Ara *et al.* (1994) demonstrated that TPZ could itself behave as an uncoupler of oxidative phosphorylation, which could lead to a drop in the available ATP pool. Elwell *et al.* (1997) studied the changes associated with long term adaption to aerobic TPZ toxicity and found increased activities in manganese superoxide dismutase and gluathione reductase, both of

mitochondrial origin. This suggests that in aerobic cells, mitchondria may be a primary target for superoxide-mediated toxicity, as well as a potential site for TPZ radical detoxification.

2.4.3 Cytosolic metabolism

DT-diaphorase has been shown to contribute significantly to the NADPH supported reduction of TPZ (under N_2) (Wang *et al.*, 1993) in both murine SCCVII (28%) and the human HT1080 (41%) sarcoma cell line in vitro. It has been suggested that TPZ may be directly reduced to its 2- and 4-electron reduction products, SR4317 and SR4330 (detoxification) by obligate two-electron reductases such as DT-diaphorase. Its proposed detoxification role is consistent with the observation that NADPH-supported TPZ radical formation in rat liver microsomes (under N_2) is unaffected by dicoumarol (100 μM) (Lloyd et al., 1991), a potent and relatively specific inhibitor of DT-diaphorase. Rat Walker tumour cell sonicates, a rich source of DT-diaphorase, readily reduce TPZ to both the 2 and 4-electron reduction products under air in the presence of NADH (Walton and Workman, 1990; Riley and Workman, 1992). This reduction is strongly inhibited by dicoumarol (100 μM). However Riley and colleagues (1993) subsequently showed that murine DT-diaphorase was not significantly involved in the anaerobic microsomal reduction of TPZ, implied not only by dicoumarol inhibition (10 μM), but also the marked oxygen sensitivity of reduction and the resultant metabolite profile.

This apparently limited role for murine DT-diaphorase was extended to human DT-diaphorase when Patterson *et al.* (1994) failed to observe a relationship between DT-diaphorase activity and either aerobic or hypoxic TPZ sensitivity in a panel of 18 human lung and breast carcinoma cell lines. The apparent inability of the 5000-fold range in DT-diaphorase activity to influence the 12-fold range in TPZ sensitivity in vitro, together with the lack of NAD(P)H dependent SR4317 formation under aerobic conditions, strongly argued against a significant role for human DT-diaphorase in the cytoprotection from TPZ exposure. Similar findings were reported by Robertson *et al.* (1994). Further support comes from work on two human colon carcinoma cell lines; HT-29 with high DTD activity and BE with undetectable DTD due to a polymorphism in the NQO1 gene (Traver *et al.*, 1992). The cytotoxicity of TPZ was essentially identical, which strongly contrasted that seen for other known DTD bioreductive drug substrates (Plumb & Workman, 1994). Utilising the human lung adenocarcinoma cell line A549, Elwell et al. (1997) demonstrated only a modest protection from aerobic (but not hypoxic) TPZ exposure by dicoumarol (2mM). This apparent "oxygen-dependence" for cytoprotection by DT-diaphorase is also suggested in studies with freshly isolated rat hepatocytes. Silva and O'Brien (1993) found

that dicoumarol (10 μM) had no effect on the rate of TPZ metabolism by hypoxic (<0.1% O_2) hepatocytes, which contrasted a later report showing significant protection by dicoumarol (25 μM) against TPZ cytotoxicty under 1% O_2 (Khan and O'Brien, 1995). However these observations may be artifacts, since dicoumarol can have multiple effects on cellular metabolism, including the inhibition of b_5 reductase and stimulation of xanthine oxidase and xanthine dehydrogenase activities (Ross *et al.*, 1993; Hodnick & Sartorelli, 1993; Gustafson & Pritsos, 1992).

The cytosolic molybdoflavoproteins xanthine oxidase and aldehyde oxidase are also known to catalyse the reduction of N-oxides (Kitamura and Tatsumi, 1971; Bickel, 1969). Purified buttermilk xanthine oxidase, supported by its cofactor hypoxanthine has been shown to efficiently reduce TPZ to SR4317, and an allopurinol-inhibitable cytosolic reductase is seen in rat and mouse liver preparations (Walton *et al.*, 1989; Walton and Workman, 1990). Further, xanthine oxidase can produce single strand breaks in plasmid DNA when co-incubated with TPZ under anoxia (Laderoute *et al.*, 1988). Liver cytosol-dependent SR4317 formation displayed an equal dependence on NADH and NADPH, but was 40-fold slower than in equivalent microsomal preparations. Cytosolic aldehyde oxidase from mouse liver preparations has also been shown to reduce TPZ in a limited fashion (Walton and Workman, 1990), but no activity was seen in rat liver preparations (Walton *et al.*, 1989). In agreement, Wang *et al.* (1993) reported that neither xanthine oxidase nor aldehyde oxidase supported significant reduction of TPZ in SCCVII and HT1080 cell line lysates (less than 10%).

2.4.4 Nuclear metabolism

A dominant role for nuclear xenobiotic-metabolising enzymes in the reductive activation of TPZ has been suggested, due to their proximity to cellular DNA (Cahill and White, 1990; Brown, 1993). Since the endoplasmic reticulum and nuclear envelope are morphologically continuous, with each sharing certain biochemical similarities, it is not surprising that both are found to exhibit qualitative similarities in enzyme content. A number of drug-metabolising enzymes have been localised in liver nuclei or nuclear envelope preparations; these include cytochrome P450 reductase, cytochrome P450, cytochrome b_5 reductase, cytochrome b_5, DT-diaphorase, UDP glucuronyl transferase, epoxide hydrolase and flavin-containing monooxygenase (Franke *et al.*, 1970; Kasper, 1974; Bornstein *et al.*, 1979; Thomas *et al.*, 1979; Patton *et al.*, 1980; Sum and Kasper, 1982). Quantitative comparison of whole nuclei, nuclear envelopes and liver microsomes has shown that considerable activity is associated with this sub-cellular compartment (Moody *et*

al., 1988). For example, utilising rat liver, Moody and colleagues found that 12% and 17% of total microsomal P450 reductase, and cyp450 activity respectively were detected in nuclear extracts. This was somewhat greater, but broadly in line with other reported values for comparative activity in whole nuclei *vs.* microsomal fractions, being 3.6 and 6.1 % for P450 reductase and 2.7 and 3.3% for CYP450 (Jernstrom *et al.*, 1976; Sagara *et al.*, 1978). In support, Hishinuma *et al.* (1987) have immunohistochemically detected nuclear envelope-associated cyp450 components in cultured rat hepatocytes. Some cyp450 immunoreactivity has been identified within the nucleus itself (Bresnick *et al.*, 1979), although others have found expression to be largely restricted to the nuclear envelope (Matsuura *et al.*, 1978; Patton *et al.*, 1980; Hishinuma *et al.*, 1987). The potential importance of outer- versus inner- nuclear localisation of CYP450 in localised drug activation has not been defined.

Cahill and White (1990) found that ≈ 5% of total microsomal P450 reductase activity was detected in the nuclei of rat primary hepatocytes, which were able to reduce TPZ to SR4317 at ≈ 4% the rate for microsomes. Important corroborative evidence has recently been reported by Evans *et al.* (1998), who showed that not only was ≈ 20% of SR 4317 formation attributable to nuclear metabolism in the human lung adenocarcinoma cell line A549, but the addition of nuclear matrix extracts to naked plasmid DNA could produce single-strand breaks under anoxia, requiring the presence of both cofactor and TPZ. Furthermore, essentially all of the DNA damage induced by hypoxic TPZ exposure in intact A549 cells, could be produced in isolated nuclei. These results point to a dominant role for nuclear-matrix reductases, assuming the intra-nuclear concentrations of TPZ in intact cells and isolated nuclei are similar. Interestingly, very little P450 reductase activity was detected in these isolated nuclei.

2.4.5 Enzyme profiles of the human tumour cell line xenografts.

Limited data exists on reductive enzyme profiles of human xenograft models. Studies in human colon and breast xenografts have identified CYP450 2A protein expression (not CYP450 2A6). Additionally in colon tumour xenografts very low levels of CYP450 2B and 2C8 were detected using the highly sensitive ECL detection method (Smith *et al.*, 1993). Evaluation of CYP450, P450 reductase and DT-diaphorase activities both *in vitro* and *in vivo* xenograft models for the panel of human tumour cell lines showed that CYP450 1A1, 1A2 and 2B activities were undetectable in all cases whereas CYP450 3A activity was elevated 2 - 10 fold in lung and breast tumour xenografts. Recent studies have suggested that elevated CYP450 3A4 and 3A5 expression is associated with increased cellular differentiation *in vitro* (Sérée *et al.*, 1998). Increases in

reductive drug metabolism in xenograft models compared to *in vitro* culture have been reported (Hejmadi *et al.*, 1996). In contrast to CYP450 3A, P450 reductase activity was reduced *in vivo* when compared to *in vitro* samples, while for DT-diaphorase apparently similar activities were recovered (Table 1). This contrasts studies conducted by Collard *et al.* (1995), who showed that DT-diaphorase activities seen in xenografts were suppressed relative to parallel *in vitro* cultures in 8 of 9 tumour cell lines.

2.4.6 Rodent solid tumour models.

Chemical induction of rodent hepatic neoplasia and its consequences upon cyp450 expression is probably the most extensively studied model *in vivo*. With few exceptions, expression of cyp450 has been found to be reduced, or absent, in tumours compared to the surrounding normal tissues. Farber and colleagues generated four different rat models for liver carcinogenesis and compared the biochemical pattern in the hepatocyte nodules to the surrounding normal tissues (Farber, 1984, Roomi *et al*, 1985). They showed a common pattern with an overall reduction of cyp450 and other phase I enzymes in the nodules. These levels were consistent with those published by other groups. Buchmann *et al.* (1985) immunohistochemically demonstrated that the level of four cyp450 forms varied widely in pre-neoplastic islets but were diminished in neoplastic nodules compared to the surrounding normal tissues. In the same model, P450 reductase was unchanged or low in islets but also reduced in neoplastic nodules. Both Buchmann and Farber suggested that these biochemical changes may contribute to the development of liver cancer. Comparable reductions in P450 reductase and cyp450 1A1 expression have been observed in induced lung tumour models (Forkert *et al.*, 1996).

2.4.7 Relevence of CYP450 and P450 reductase to TPZ cytotoxicity *in vivo*.

Some human P450 species have notably different catalytic activities towards various substrates, than that predicted by studies in animal systems. Consequently, extrapolation of metabolism data from rodent P450s to their human orthologues is hazardous. Large species-specific differences in the enzymes involved in drug metabolism have been observed (Gonzalez, 1992). In contrast, P450 reductase is more cleary implicated in the bioactivation of TPZ in both human and rodent tissues. Similarities might be anticipated from the close sequence homology between species (Nebert *et al.*, 1991), and the fact that P450 reductase requires neither substrate binding nor conformational changes to facilitate the transfer of an electron to TPZ. Thus it would

be anticipated that the overall reduction reaction would be strongly dependent upon the redox potential of TPZ and the thermodynamics of the system, as opposed to reductive mechanism of the CYP450s, where active site constraints would contribute to specificity.

An appreciation of which one-electron reductases are dominant in the activation of TPZ in human tumour tissues, would aid the clinical development of TPZ through the rational selection of tumour types. This might also allow the logical application of enzyme-profiling studies conducted on large panels of human cancer cell lines (Fitzsimmons *et al.*, 1996), where patterns of reductive enzyme expression in particular tissue types can be examined in more detail *in vivo*. Further, it would also allow one to predict potential normal tissue toxicities, such as hepatotoxicity associated with ethanol-induced 2E1, or the relatively high P450 reductase levels found in kidney medulla. Both these organs would be anticipated to have regions of low pO_2.

A good working knowledge of TPZ enzymology might also aid in the development of novel benzotriazene-di-*N*-oxide analogues with superior oxic/hypoxic differentials (Zeman *et al.*, 1989). Structure-activity relationships might ultimately be predicted from active site modelling of characterised CYP450 isozymes, or from variations in redox potential and the concurrent changes in rates of reduction by P450 reductase (Butler and Hoey, 1993b). The rational design and development of novel anologues through enzyme-directed techniques would be an attractive strategy.

The observation that cyp450 2E1 may participate in TPZ cytotoxicity (Khan and O'Brien, 1995) may have important implications for the acute liver toxicity that is seen in rat models (White *et al.*, 1992). TPZ-associated liver necrosis is confined to hepatocytes in the pericentral zone, a region that only experiences oxygen tensions of 2 - 4% (Lemasters *et al.*, 1981). Furthermore, it has been suggested that ethanol-induction of CYP450 2E1 in the centrilobular region of the liver may participate in the accentuation of the oxygen gradient between the perivenous and centrilobular regions of the liver (Lieber, 1984; 1990). The hypothesis that increased oxidase activity of CYP450 2E1 in the centrilobular region results in increased oxygen consumption while also increasing oxygen radical production, would account for the regioselective hepatotoxicity observed following ethanol treatment. While the importance of oxygen tension on TPZ-induced rat hepatocyte toxicity has been examined in vitro (Costa *et al.*, 1989), the potential impact of ethanol consumption on TPZ related hepatotoxicty in humans is unknown.

2.4.8 Predicting clinical response.

In view of the uncertainty surrounding the enzymology of TPZ, predictive assays such as the alkaline 'comet' single cell gel electrophoresis technique, which measures the level of DNA ssb in individual cells following TPZ treatment, may be the most appropriate way forward at present (Olive and Durand, 1992; Olive *et al.*, 1993; Olive, 1995). Using the functional endpoint of DNA damage as a predictive marker, a measure of both the oxygen tension and the activity of single-electron reductases within individual cells is determined. This overcomes the difficulty in knowing how to interrelate independent measurements of enzyme activity with oxygen tension. Moreover, a knowledge of the possible influences of subcellular locatisation of key reductases at different oxygen tensions becomes unimportant.

The utility of this approach has been demonstrated *in vitro* and *in vivo* (Olive *et al.*, 1996; Simm *et al.*, 1996; 1997), and has confirmed that DNA ssb correlate with cytotoxicity in both human and murine cell lines and xenograft models. Olive and collegues (1996) found the comet assay to be an adequate predictor of surviving fraction in xenograft models if samples were collected within 1 hour of TPZ administration. A similar relationship between DNA ssb at 60 minutes and cell survival were reported by Simm and colleages (1996) in a murine cell line in vitro. These relationships were measureable at clinically relevent doses of TPZ, with the high levels of DNA ssb formation allowing accurate detection, yet with the background DNA damage being comparatively minimal. However variations in the cellular ability to repair DNA damage could complicate interpretation (Keohane *et al.*, 1990; Beiderman *et al.*, 1991), and the apparent inability of the technique to predict interactive toxicity with radiation may prove to be a limitation (Siim *et al.*, 1997).

Simm and colleagues (1997) demonstrated a relationship between tumour oxygenation, determined by the immunodetection of binding of the pentafluorinated etanidazole derivative EF5, and DNA ssb induced by TPZ treatment. This relationship was consistent when tumour xenograft oxygenation was increased by carbogen (95% O_2, 5% CO_2) and nicotinamide administration or reduced by 10% oxygen breathing ($r = 0.732$; $P<0.001$). However only half of the variation in DNA damage could be accounted for by variations in tumour hypoxia as measured by binding of the hypoxic marker EF5, and it was suggested that the discrepancy may arise in part from the different oxygen tension dependencies for the reductive metabolism of nitroimidazoles and TPZ (Koch, 1993). However it must also be considered that the enzymology of reductive activation of these two class of compounds also differs significantly (Walton and Workman, 1987; Walton *et al.*, 1989), making it difficult to differentiate between differences in oxygen dependence and

enzyme profiles. Ultimately a clearer knowledge of the key reductive enzymes responsible for TPZ metabolism in humans could allow targeting of this drug to patients that have tumours with favourable enzyme profiles and pO_2 dependence. This together with non-invasive tumour imaging techniques (i.e. ^{19}F MRI) may aid in predicting the response of individuals to TPZ before treatment. Such an approach, if viable, would have considerable advantages over the intrinsically invasive nature of tissue sampling necessary for comet assay measurements.

Chapter 3

3. Overexpression of human NADPH:cytochrome P450 reductase in the breast adenocarcinoma cell line MDA-231 confers enhanced sensitivity to the bioreductive agent, tirapazamine *in vitro*.

3.1 Introduction

Following the observations in the panel of breast cell lines, a mammalian expression vector containing human P450 reductase (P450R) cDNA was transfected into the lowest endogenous P450R expressing cell line MDA-231. Since correlational studies do not imply causality, this provided a mechanistic approach to substantiate the relationship between P450R activity, SR4317 formation and tirapazamine (TPZ) sensitivity in the original panel of human breast cell lines.

3.2 Methods

Plasmid construction. The cDNA for human NADPH:cytochrome P450 reductase (P450R) was kindly provided by Professor C.R. Wolf (ICRF, University of Dundee, Scotland). Full length cDNA (2.4kb), originally isolated from human skin fibroblasts (Shephard *et al.*, 1992), was subcloned into the pTZ19R bacterial vector. Restriction enzyme digest with EcoRI and SalI (Gibco BRL) allowed the forced ligation into the multiple cloning region of the retroviral vector pBabe/Puro (Morgenstern and Land, 1990). The Moloney murine leukaemia virus LTR's promoter will drive transcription of the inserted gene, and has been demonstrated to be more efficient than most internal promoters in a number of cell types (Wilson *et al.*, 1988; Osborne and Miller, 1988). The *pac* gene under the control of the SV40 early gene promoter confers resistance to the amionacyl nucleoside antibiotic, puromycin. The ATG $\overline{gag}$ sequences necessary for the high titre characteristics of the pBabe vector, when packaged into a retroviral system, does not influence expression of an inserted gene at the level of translation.

Transfections and clonal selection of MDA 231 cells. Cells in exponential growth were harvested with a cell scraper, washed and resuspended in phosphate "cytomix" (Van den Hoff *et al.*, 1992) to increase cell survival following electroporation. $5x10^6$ cells were mixed with 10 μg linearised pBabe/Red vector and electroporated. Cells

were plated at low density and 48h later were exposed to 3 μg/ml puromycin. Selection was maintained for at least 8 weeks. Individual colonies were isolated and samples of each were grown on glass coverslips for subsequent confocal microscopic examination. Cell monolayers were washed, fixed in 1:1 acetone:ethanol, blocked with 0.1% BSA, and incubated with anti-human P450R polyclonal antibody (1:100 dilution). Anti-rabbit IgG FITC-conjugated secondary antibody was used (1:1000 dilution) to visualise the uniformity and subcellular distribution of the primary antibody binding. The pre-screening of clonal lineage's on glass coverslips by immunohistochemistry avoided the unnecessary expansion of non-expressing clones for enzyme activity assays. Positive clones were further assessed for uniformity by similar anti-P450R antibody staining using flow cytometric single-cell analysis. Those populations of apparent single cell parentage were expanded for enzyme activity analysis and subsequent TPZ sensitivity work. A large number of colonies were screened in order to generate a set of clones exhibiting a range of elevated P450R expression.

Cell lysate and primary breast biopsy sample preparation. Cell lysates were prepared in an identical manner to that previously described (Chapter 2). However the fractionation technique for the breast tumour biopsy samples was different to the standard S-9 lysate preparation. Tissue was cut up with surgical scissors under liquid N_2, homogenised in 10mM HEPES, 1mM EDTA, 0.5mM benzamide, 0.5mM PMSF, 1.0 μg/ml trypsin inhibitor (pH 7.4, 4°C) at a ratio of 1 gram tissue : 20ml buffer, and sonicated for 3 x 5 sec. (23kHz) as described previously. An initial 1600g spin (4°C) was used to remove excess cellular debris and the resulting supernatant was spun at 105,000g for 45 mins., (2°C). Membrane pellets were dried and resuspended (with the aid of homogenisation) in Tris buffered saline (pH 7.4) containing 20% glycerol. In order directly to compare the P450R activities seen in the clinical tumour biopsy samples with the *in vitro* cell lines, identical membrane preparations were performed utilising a representative set of both the original panel of breast cell lines and the P450R-transfected MDA 231 clonal lines.

Tirapazamine sensitivity. Dose-response curves were determined under aerobic and hypoxic conditions, and IC_{50} values independently collated as described in chapter 2. Drug exposures were 3 h under hypoxic and 3 or 96 h under aerobic conditions. Total growth time before assay with MTT was 96 h. Experimental details differ from those described in chapter 2 (Patterson *et al.*, 1995b), in that all hypoxic exposures were conducted under conditions of catalyst-induced anoxia (< 1 p.p.m. O_2). All plastics and media were

preincubated in anoxia for 24 h before use to remove residual O_2. The values of IC_{50} in wild-type MDA 231 cells treated with TPZ under these extremely hypoxic conditions did not differ significantly from the earlier method of continual anoxic gas flow. Metabolism studies were conducted as described in chapter 2. Statistical analysis of the data was conducted as before.

3.3 Results.

Clonal characterisation. Six stable clones were selected, representing 6 to 53 -fold elevations in P450R activity above the parental cell line. These activities are thus within and beyond the range seen in the original panel of six breast cell lines.

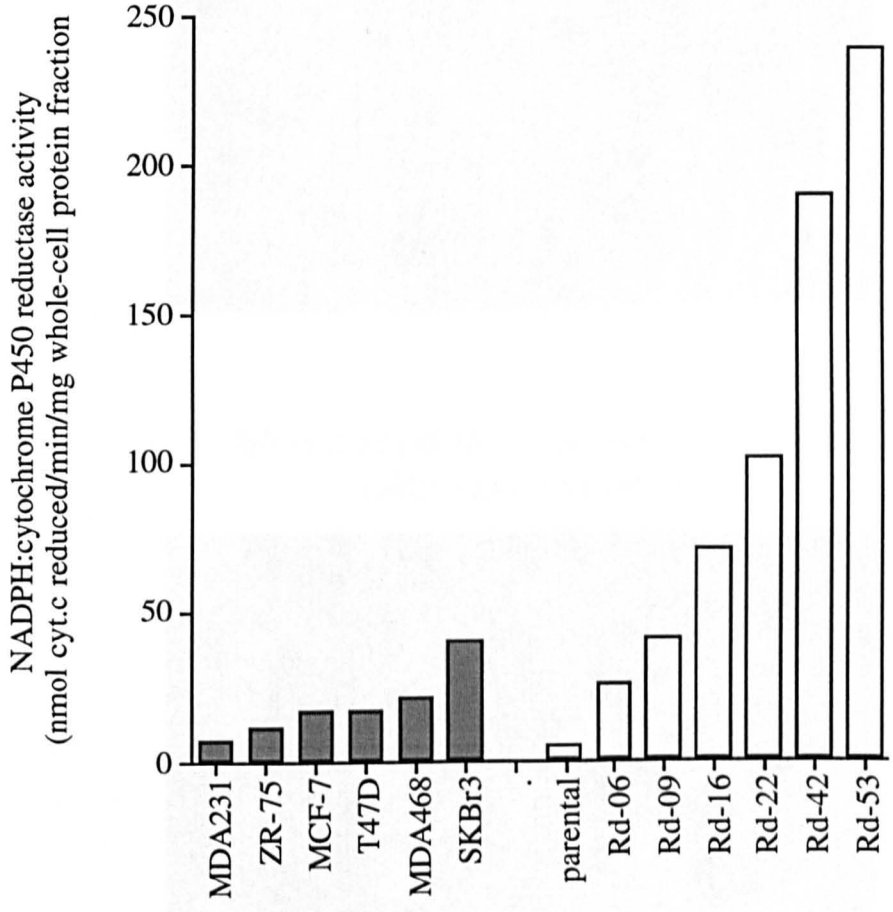
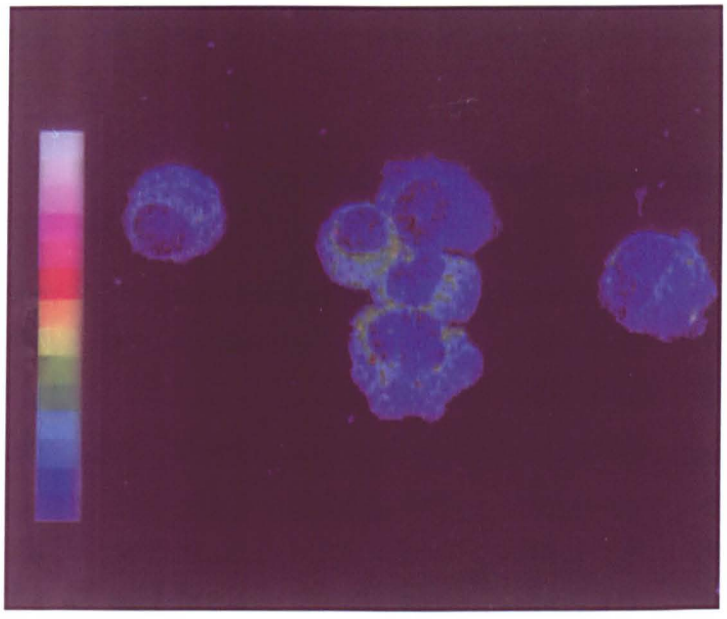


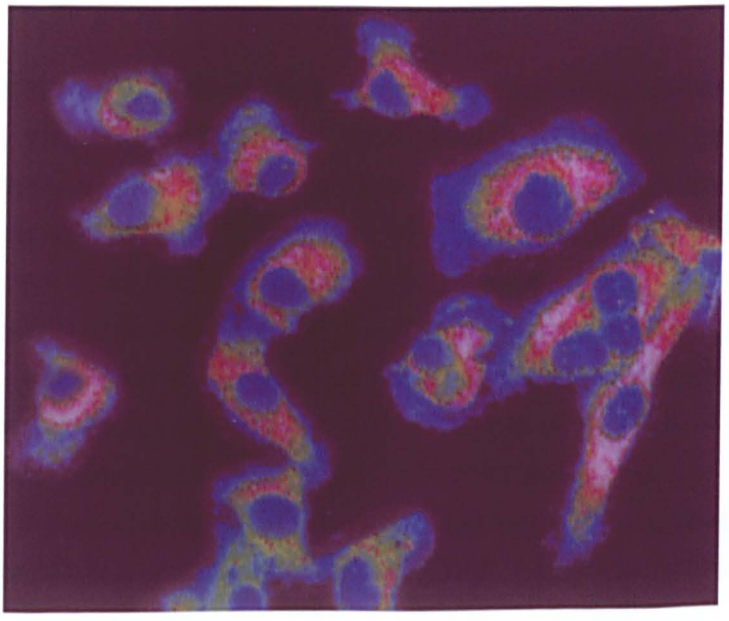
Figure 1: Comparative NADPH:cytochrome P450 reductase (P450R) activities in a panel of six human breast cancer cell lines, and a series of clonal lineages of the lowest P450R-expressing cell line, MDA 231, into which human P450R has been stably transfected.

FIG 2: CONFOCAL MICROSCOPIC IMAGE OF IMMUNOREACTIVITY OF FITC-LABELLED ANTI-P450 REDUCTASE.

Parental MDA231



Mixed population of stable clones following selection



Confocal visualisation identified the presence of P450R protein which was found to localise to the endoplasmic reticulum (figure 2). Western blot experiments confirmed the expression of a single immunoreactive protein of identical gel mobility to that of the endogenous P450R protein (~78 kda) (data not shown).

DT-diaphorase and NADH:ferricytochrome b_5 reductase activities, total glutathione, and cell doubling times determinations were also conducted on the six clonal lines. No statistically significant differences were seen for any of these parameters. The data is summarised in table 1.

Table 1: MDA 231 P450 reductase transfected cell lines: determination of key characteristics for each clonal lineage.

Cell line	P450 reductase ± s.d. nmol $min^{-1}mg^{-1}$	B_5 reductase ± s.d. nmol $min^{-1}mg^{-1}$	DT-diaphorase ± s.d. nmol $min^{-1}mg^{-1}$	Intracellular GSH ± s.d nmol 10^{-6} cells	Doubling time ± s.d. (h)
Parental	4.5 ± 2.4	42.9 ± 5.2	36.6 ± 35.3	3.6 ± 3.0	23.0 ± 3.0
Rd-06	25.3 ± 3.6	56.3 ± 6.2	7.8 ± 2.1	4.3 ± 1.5	22.7 ± 3.7
Rd-09	40.6 ± 7.8	40.1 ± 2.4	37.8 ± 14.3	2.4 ± 1.9	24.0 ± 2.9
Rd-16	71.2 ± 5.4	39.4 ± 2.4	9.5 ± 7.9	5.7 ± 1.6	22.2 ± 3.7
Rd-22	100.1 ± 17.1	36.2 ± 8.6	6.3 ± 2.4	5.0 ± 2.2	23.3 ± 1.9
Rd-42	189.6 ± 31.6	42.3 ± 4.7	12.1 ± 13.2	1.9 ± 0.4	24.6 ± 3.4
Rd-53	239.0 ± 19.7	45.1 ± 5.4	36.3 ± 25.9	3.8 ± 1.3	23.9 ± 4.1

Tirapazamine sensitivity. Analysis of the sensitivity of each of the derived clones to TPZ was conducted, and IC_{50} values were determined as described (table 2). A representative dose-response curve is shown in figure 3. The data demonstrated a clear relationship between elevated P450R activity and increased sensitivity to tirapazamine under 3h hypoxic (slope = -1.83 ±0.26; P=0.0019) and 3h aerobic (slope = -1.52 ±0.30; P=0.0041), or 96h aerobic (slope= -1.38 ±0.26; P=0.0030) exposure conditions (Figures 4 & 5 respectively). Furthermore, the rate of formation of the 2-electron reduction product, SR4317, using cell lysates incubated with TPZ and NADPH under hypoxic conditions, supports the conclusion that P450R is responsible for the reductive metabolism of TPZ in this *in vitro* model (Figure 6). It can be seen from these data that there is a strong correlation (slope value = 3.31 ± 0.43; P = 0.0015) between P450R

activity and SR 4317 formation, with higher values of enzyme activity resulting in greater rates of metabolism. When similar experiments were carried out in air, no metabolism was detected.

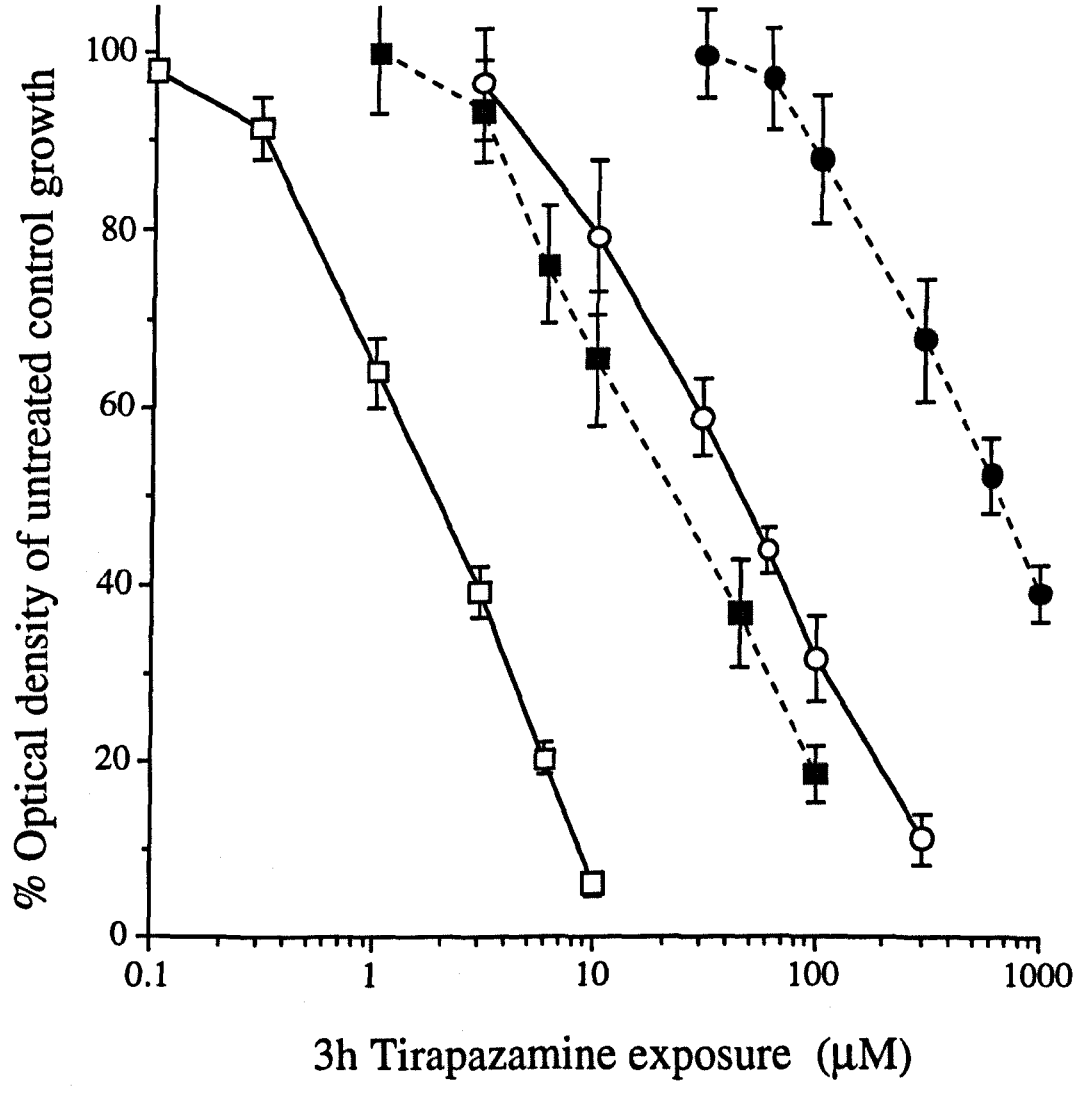
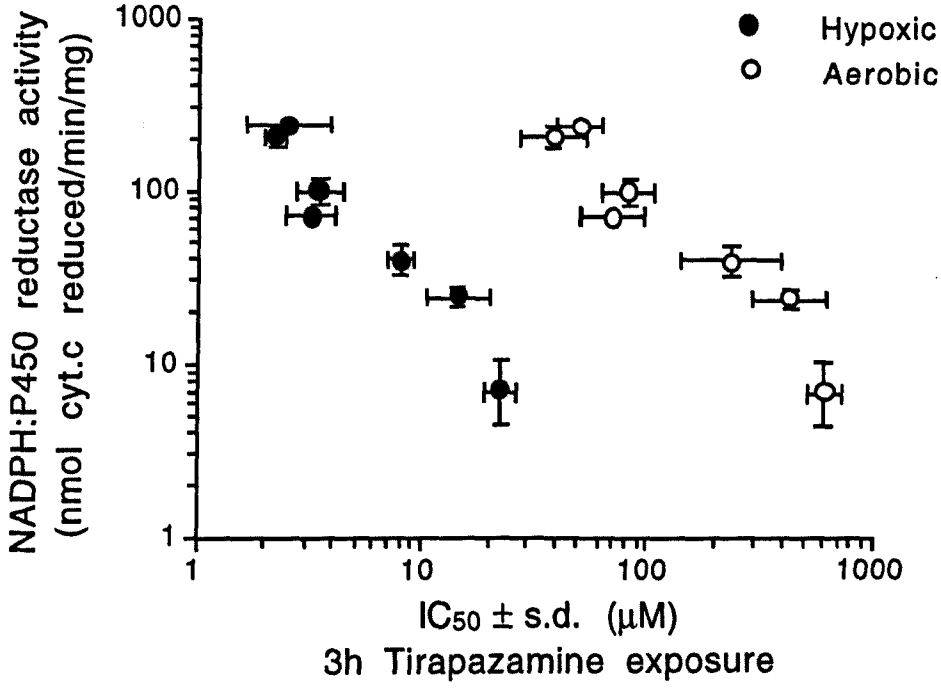
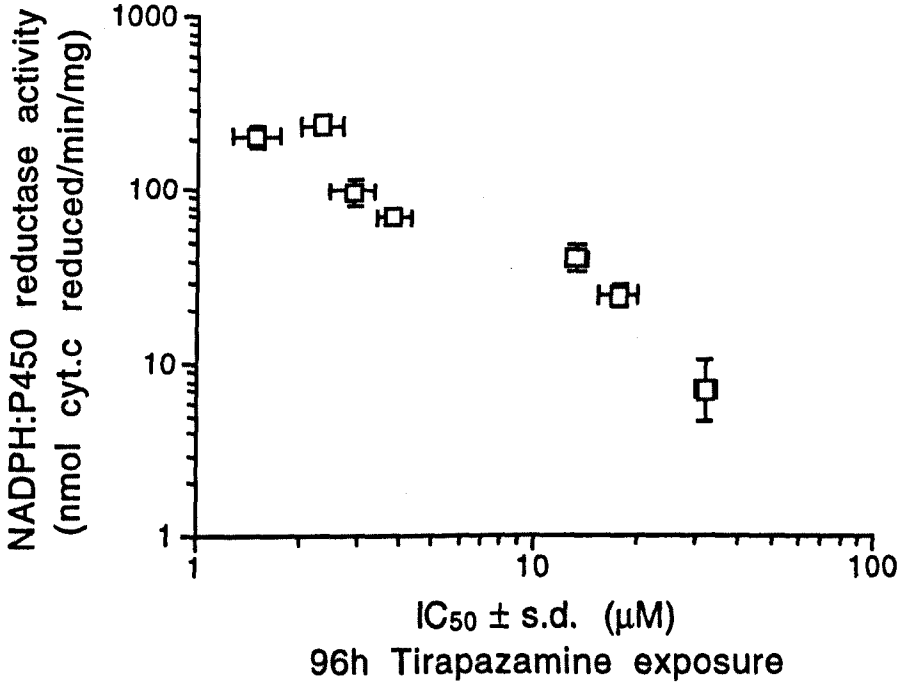


Figure 3: Comparative dose-response curves for 3h aerobic (circular symbols) and 3h hypoxic (square symbols) TPZ exposures in the parental MDA 231 breast adenocarcinoma cell line (closed symbols), and a clonal line Rd-42 (open symbols), stably over-expressing human NADPH:cytochrome c (P450) reductase. Relative P450 reductase activities are 4.5 and 190 nmol cyt c. reduced min^{-1} mg^{-1} protein respectively, representing a 42-fold elevation in whole-cell actvity.





Figures 4 & 5: Dependence of IC_{50} values of the MDA 231 clones exposed to tirapazamine for 3h under aerobic (O) or hypoxic (●) conditions (fig.4), or for 96h under aerobic (□) conditions (fig.5), on P450 reductase activity. Bars indicate standard deviations.

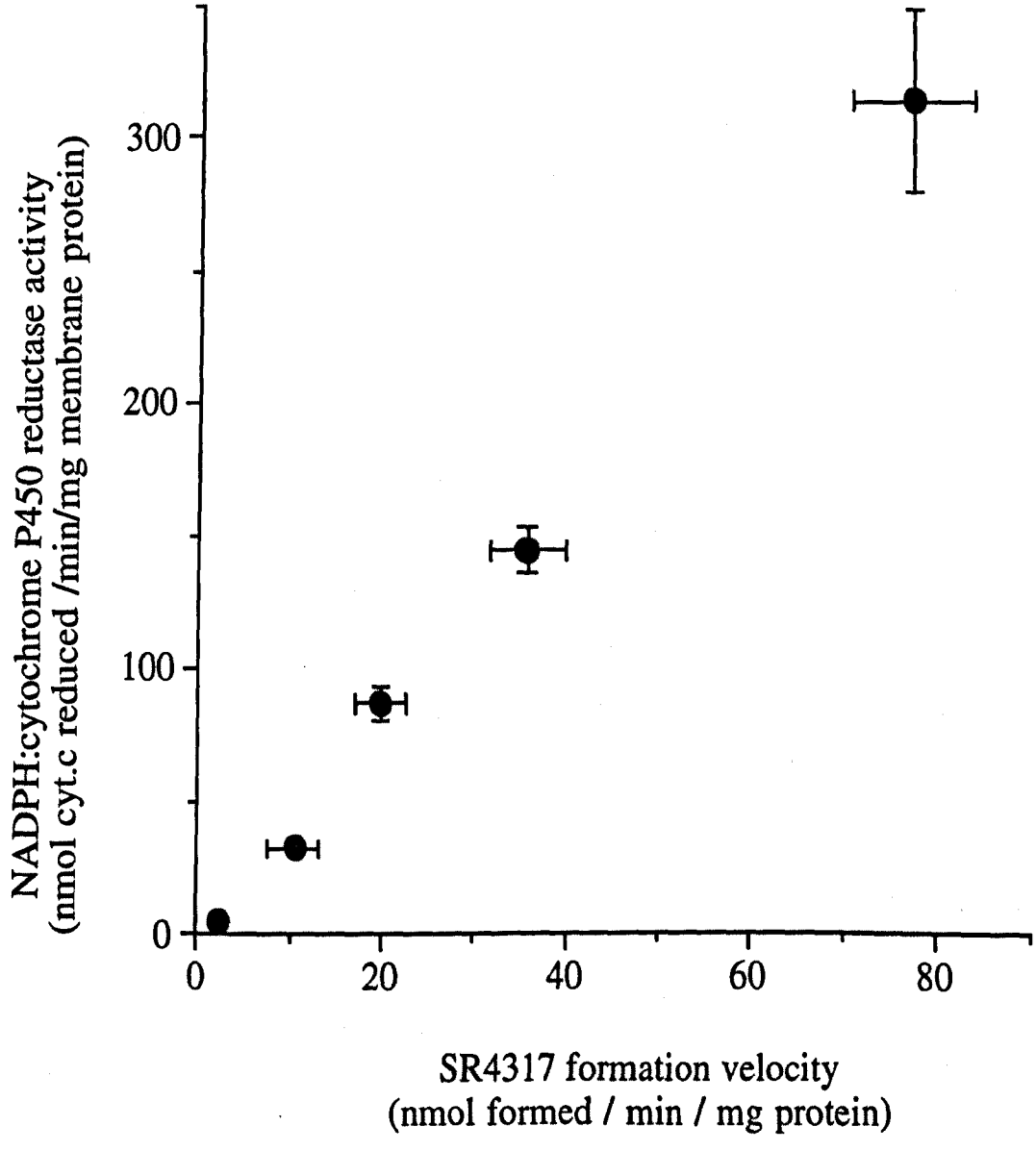


Figure 6: Dependence of P450 reductase activity for the ability of membrane-preparations from the transfected MDA 231 clones to convert TPZ to SR 4317 under hypoxic conditions. Bars indicate standard deviations.

Table 2: MDA 231:P540 reductase transfected breast cancer cell lines: response to TPZ under aerobic or hypoxic conditions.

Cell line	Tirapazamine IC_{50} (μM) ± s.d.			Differential toxicity
	96-h Aerobic exposure	3-h Aerobic exposure	3-h Hypoxic exposure	IC_{50} ratio of 3-h oxic/hypoxic
Parental	34.2 ± 3.9	615 ± 104	22.6 ± 3.6	25.8
Rd-06	18.6 ± 5.6	329 ± 45	15.1 ± 4.6	21.8
Rd-09	13.6 ± 1.8	258 ± 120	8.1 ± 1.0	31.9
Rd-16	4.0 ± 1.3	72.6 ± 20.0	3.3 ± 0.8	22.0
Rd-22	3.1 ± 1.3	84.4 ± 21.1	3.5 ± 0.8	24.1
Rd-42	1.5 ± 0.4	40.7 ± 13.1	2.1 ± 0.6	19.4
Rd-53	2.4 ± 0.8	50.6 ± 12.3	2.6 ± 1.0	19.5

Comparative activity of P450 reductase in human breast cancer cell lines, the transfected clonal lines and clinical breast tumour biopsy samples. Five of the panel of original breast cancer cell lines and four of the isolated clonal lines (Rd-06, Rd-09, Rd-16 and Rd-42) were used to generate both whole-cell lysates (S-9) and whole-membrane fractions. As whole-membrane fractions are routinely prepared from all surgical breast biopsy samples for receptor analysis in other studies, membrane samples were prepared in an identical from the panel of breast cell lines and four of the P450R clonal lines. The activity of P450R for both preparator methods, in each clonal line, correlates well ($P = 0.007$), with membrane fractions showing an approximately two-fold higher level of activity (per mg of protein) than the whole-cell lysates. The membrane fraction P450R activities of each group of samples is plotted in figure 7. The activities found in the range of P450R clonal lines is within and beyond that seen for both the in vitro cell lines and the breast biopsies. A clear heterogeneity in functional P450R activity is seen in the breast biopsy samples, with an 18-fold range in membrane-associated activity. Indeed, within this limited group, 4 of 14 samples have activities greater than those found in the panel of unmodified breast cell lines. Further, immunohistochemical analysis of a range of sections taken from each tumour biopsy demonstrated uniform immunoreactivity localised

to tumour epithelia. In some cases P450R-immunoreactivity appeared greater in peripheral normal epithelia than the adjacent neoplasia (Figure 8).

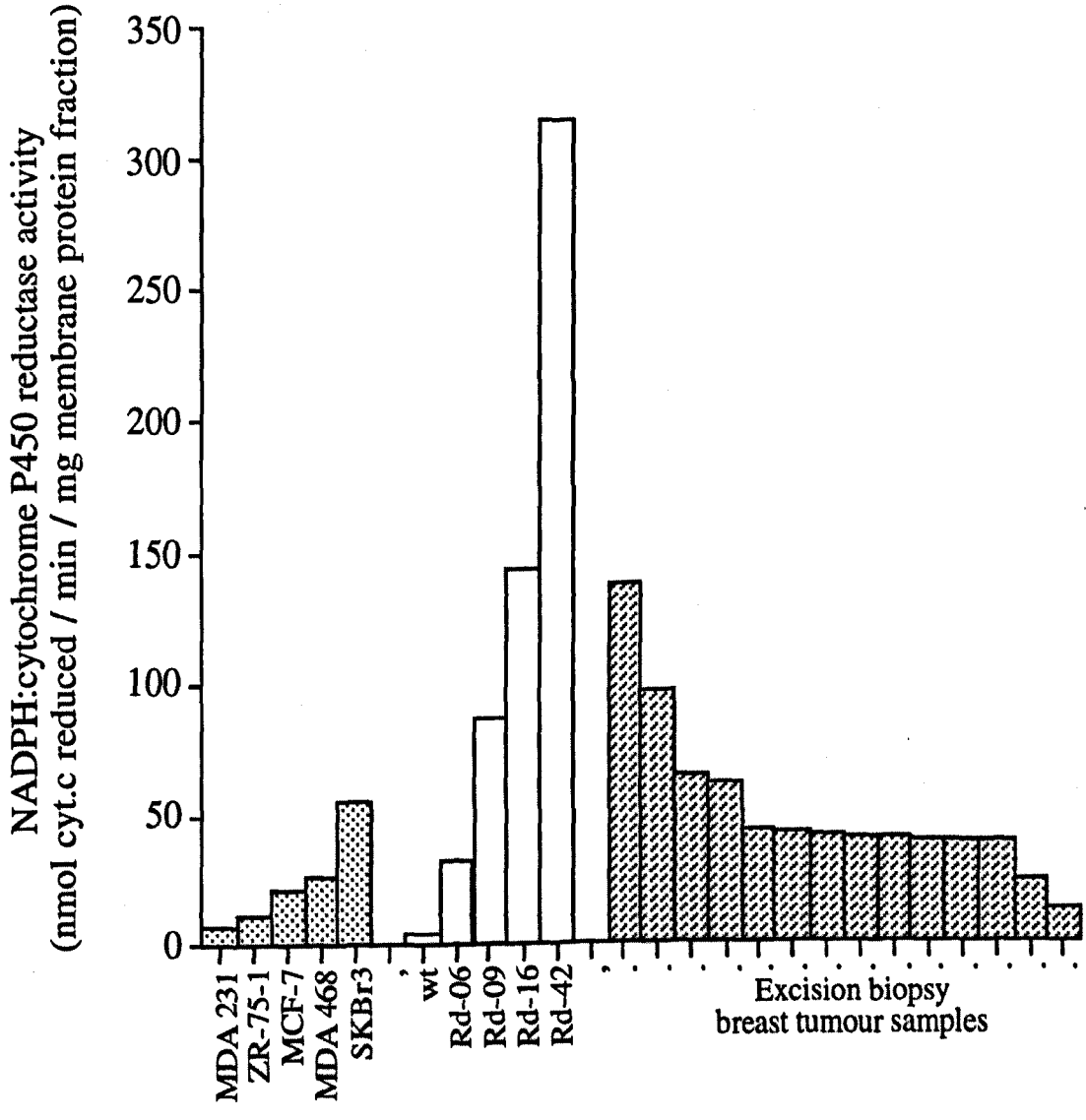


Figure 7: P450 reductase activity in human tumour cell lines, transfected MDA 231 clones and biopsy samples from primary human breast tumours.

FIG 8: HUMAN BREAST CARCINOMA EXPRESSION OF NADPH: CYTOCHROME P450 REDUCTASE

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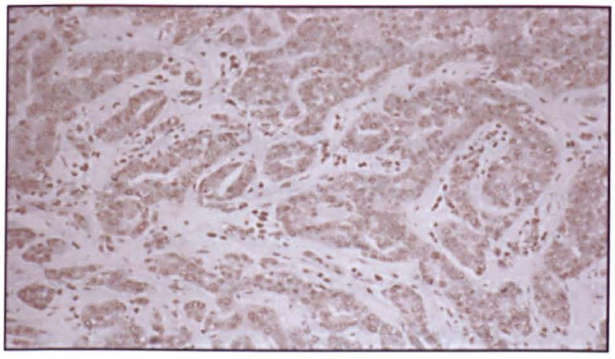

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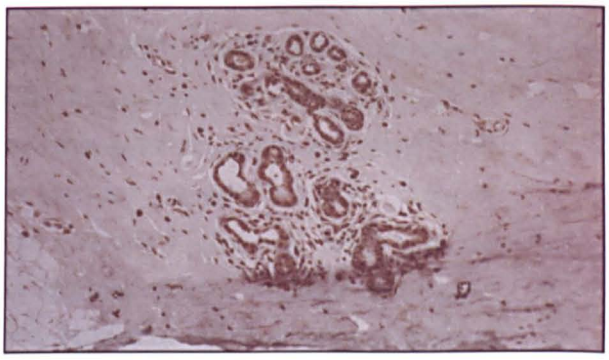

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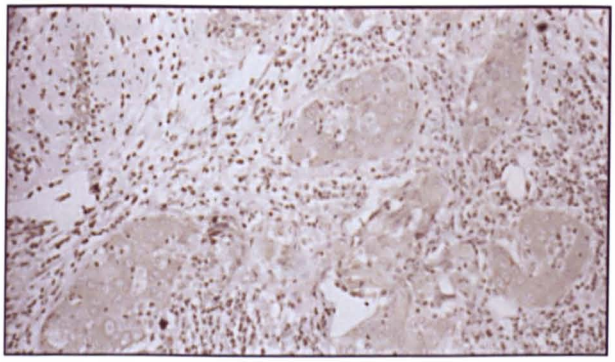

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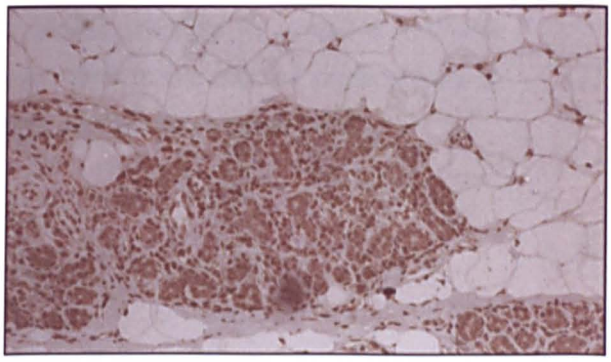

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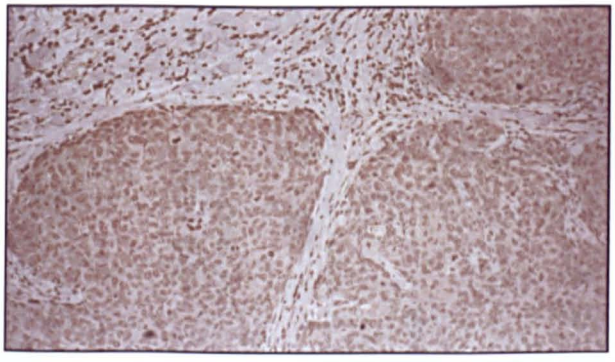

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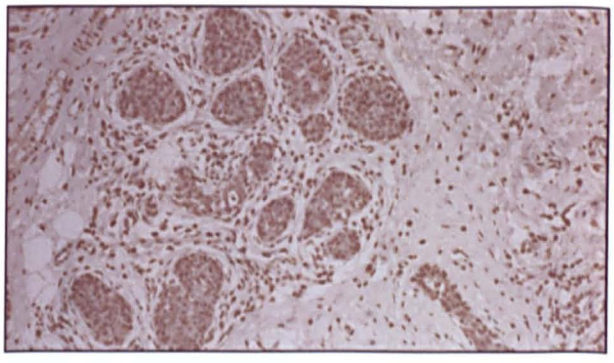
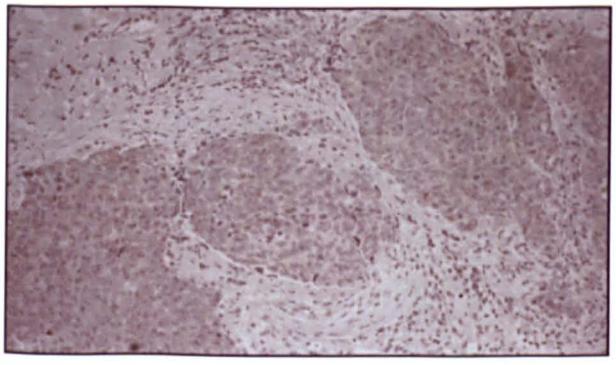


Patient no. 3822 pre-immune serum



Paired sections of three typical breast neoplasial (above left) and their adjacent histologically normal ductal tissues (above right). Immunoreactivity is apparently down-regulated in carcinoma compared to paired normal tissues. Expression appears to be localised to epithelium, with no detectable stromal staining.
No elevated epitelial staining is seen in sections incubated with control pre-immune serum (opposite).

Establishing the Rd-16 clonal cell line as a xenograft model in nude mice. During extended periods of selection-free *in vitro* growth, Rd-16 was found to express stable levels of P450R activity. This lineage was used to establish intraperitenial xenografts in the lower back of nude mice, in order to evaluate its potential as an *in vivo* model of P450R overexpression. Four xenografts were initially established, from one of which, a tissue sample was serial passaged to create the next passage of xenografts and so forth for 4 passages. *In vivo* samples were harvested from each xenograft and the P450R activity was quantified. Initially activities were low, but by passage 3 a wide range in P450R activity was seen (figure 9), which was not obviously inversely related to the proportion of necrotic tissue in the tumour mass, as determined by immunohistochemical analysis (data not shown). Neither was enzyme activity related to the final tumour mass at the time of excision (Figure 10). More detailed histological analysis revealed the P450R immunoreactivity to be somewhat heterogeneous in the viable area of xenograft tissue sections (figure 11). This implied either a lack of clonal stability *in vivo*, or perhaps variations in the functioning of the MLV LTRs *in vivo*. Due to the lack of predicability in total P450R activity in this xenograft model, no further studies were conducted.

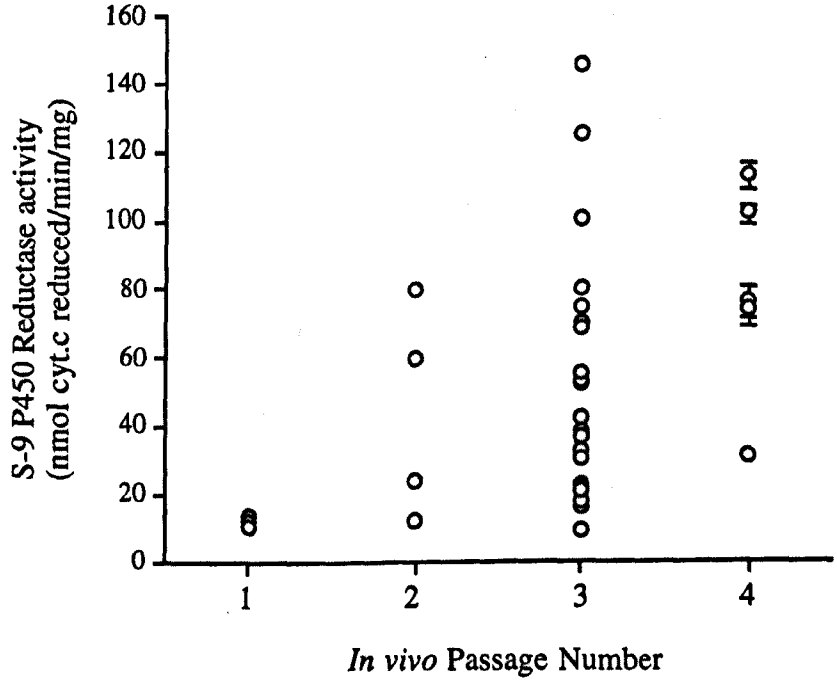


Figure 9: Individual P450 reductase determinations of Rd-16 xenografts serially passaged in nude mice *in vivo*. Mean *in vitro* activity of Rd-16 cell line = 71 ± 5 nmol/min/mg.

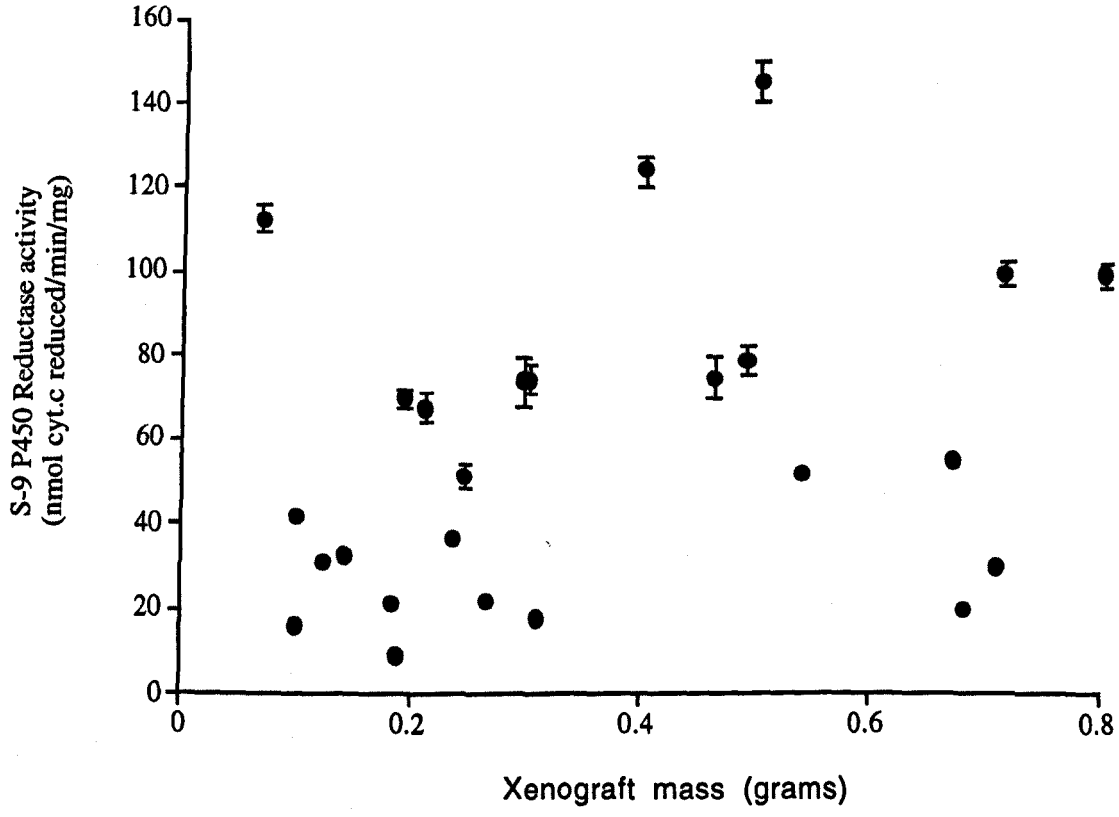
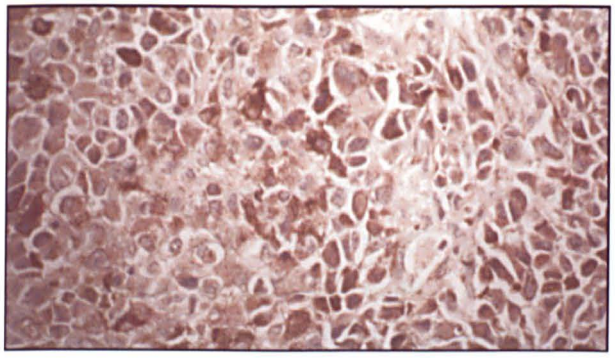


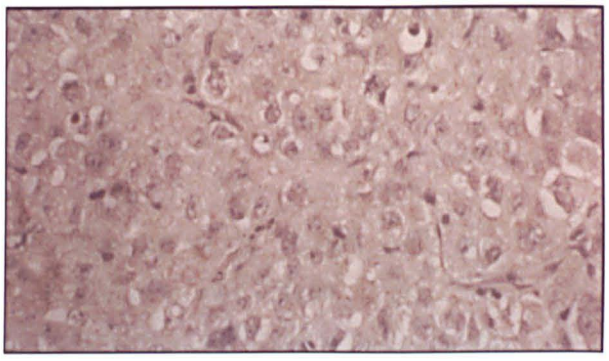
Figure 10: Relationship between P450 reductase activity and total tumour tissue mass in the Rd-16 xenograft model *in vivo*.

Figure 11: In Vivo NADPH: Cytochrome P450 Reductase Expression in the MDA-231 Parental and Rd-16 Breast Cell Line Xenografts

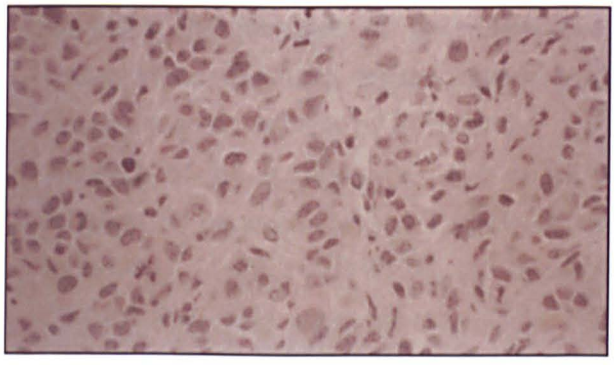
Rd-16



Parental

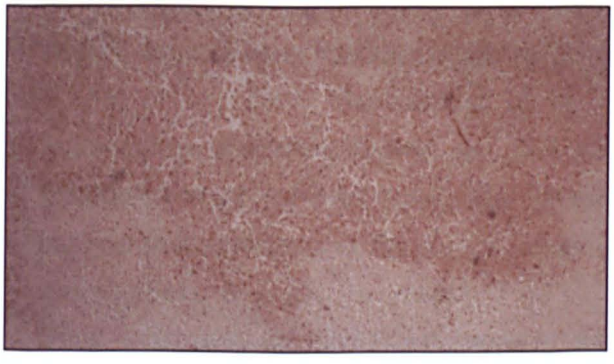


Rd-16 pre-immune serum

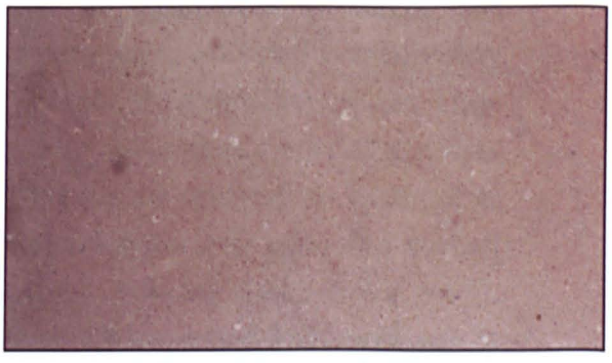


x40 magnification of MDA-231 parental and Rd-16 xenograft sections, stained for P450 Reductase expression

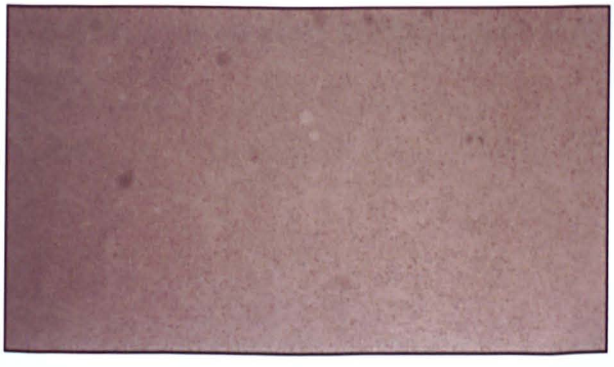
Rd-16



Parental



Rd-16 pre-immune serum



x25 magnification of MDA-231 parental and Rd-16 xenograft sections, stained for P450 Reductase expression

3.4 Discussion

Evidence from enyzme-profiling studies (chapter 2) has implicated P450R as an important determinant of the hypoxic and aerobic toxicity of tirapazamine in breast cancer cells *in vitro*.

In order to unequivocally confirm the contribution of this enzyme by genetic means, the lowest of these P450 reductase-expressing breast cell lines, MDA 231, was transfected with the full length human P450R cDNA (Shepard *et al.*, 1992), under the control of the Moloney murine leukaemia virus LTR (Osborne and Miller, 1988). Six stable clones were selected, representing 6 to 53 -fold elevations in P450R activity above that of the parental MDA 231. These activities were within and beyond the range seen in the original panel of six breast cell lines (Patterson *et al.*, 1995). Confocal visualisation identified the presence of immunoreactive protein which apparently localised to the endoplasmic reticulum, while western immunoblots confirmed the expression of a single immunoreactive protein of identical gel mobility to that of endogenous protein (78 kda). Analysis of the sensitivity of each of the derived clones to TPZ demonstrated a clear relationship between elevated P450R activity and increased sensitivity to TPZ under 3h hypoxic ($\leq$ 11-fold; P=0.0019) and 3h aerobic ($\leq$ 15-fold; P=0.0041), or 96h aerobic ($\leq$ 23-fold; P=0.003) exposure conditions. Further, the rate of formation of the 2-electron reduction product, SR4317, correlated with P450R activity, supporting the conclusion that P450R is responsible for the reductive metabolism of TPZ in this *in vitro* breast adenocarcinoma model. These observations support the conclusions of others, that P450R-dependent metabolism is proceeding *via* an oxygen-sensitive cytotoxic intermediate (Lloyd *et al.*, 1991; Fitzsimmons *et al.*, 1994). Statistical comparison of the inter-relationships of enzyme activity and TPZ sensitivity from the original six breast cell lines (Patterson *et al.*, 1995b) *versus* that of the MDA-231 clones provided no evidence to suggest that variations in the slopes for each exposure condition were other than random (3h hypoxia, P=0.19; 3h aerobic, P=0.90; 96h aerobic, P=0.54; Aspin-Welsh t-test). Thus, analysis of the data sets not only implicated the cDNA-expressed P450 reductase in the reductive metabolism of TPZ, but also indirectly substantiated the earlier conclusions (chapter 2) that the endogenous P450R activity measured in the six breast adenocarcinoma cell lines was correctly identified as the major determinant of TPZ sensitivity.

3.4.1 Relevanceof sub-cellular localisation for TPZ bioactivation.

It has been proposed that only the nuclear metabolism of TPZ is responsible for the generation of the radical species which ultimately give rise to the lethal DNA damage (Brown, 1993). Yet, over-expression of full length human P450R cDNA in a breast cancer cell line conferredmarked sensitization to both hypoxic and aerobic TPZ exposure, despite an apparent reticular localisation. However, due to the intense immunoreactivity of the rough endoplasmic reticulum, and the absence of nuclear fraction activity assays or immunohistochemical analysis of isolated nuclei, it is difficult to accurately differentiate between reticular and nuclear envelope localisation. What is clear is that no detectable increase in intra-nuclear P450R immunoreactivity was observed, although since the P450R polypeptide contains putative lysine-rich nuclear-localisation signals, such a possibility can not be excluded.

In the absence of oxygen the TPZ radical is thought to undergo an oxidising reaction with DNA (Laderoute *et al.*, 1988; Baker *et al.*, 1988) yielding a DNA radical and SR4317. This may be the first step in a chain reaction between the DNA radical and TPZ, that could result in multiply damaged sites from a single reductive event. Laderoute *et al.* (1988) demonstrated the existence of a short chain reaction during the radiolytic reduction of TPZ in a solution containing formate. Wardman *et al.* (1995) have suggested that the nitroxide anion mediated hydrogen abstraction from the sugar backbone of DNA will result in strand breakage and the formation of a sugar radical, and that this radical can subsequently reduce a further molecule of TPZ, establishing a highly localised chain reaction of TPZ activation, thereby 'amplifying' the initial DNA damage. It has been suggested that such multiply damaged sites in DNA (reminiscent of high-LET radiation damage) could be the critical lesions for TPZ -induced hypoxic cytotoxicity (Brown, 1993) since they appear to be particularly difficult to repair (Wang *et al.*, 1992).

Wardman *et al.* (1996) subsequently argued, following theoretical considerations of the competition between diffusion of the second radical away from the site of generation at the initial sugar radical and its reaction close to the original site of DNA damage, that considerable doubt must exist as to whether damage initiated by a single drug radical could lead to the clustered damage that is proposed to occur (Wang *et al.*, 1992; Brown, 1993). This conclusion was based upon calculations suggesting the radical was predicted to live for ≈ 1 to 10 msec, through which the mean diffusion distance of the radical in the cytosol was found to approximate to 0.5 - 2 μm. These estimates of radical stability under hypoxia

were consistent with earlier experimental estimates of 8 msec for the first half-life of the radical under anoxia (pH 7.4, initial TPZ concn. ≈ 4μM) by Laderoute *et al.* (1988), who concluded that "*the 'natural' lifetime of of the radical was sufficiently long that it could diffuse over significant distances within hypoxic cells and thus inflict oxidative damage on cellular targets* ". By implication microsomal-generated TPZ radicals may have just as effective an access to DNA as any nuclear membrane-generated radicals under hypoxia. However, such a diffuse "halo" of microsomal radical generation around the nucleus would still leave the postulated formation of clustered DNA damage unexplained. One might suggest that if short chain reactions between DNA radicals and TPZ are unlikely to 'amplify' damage, due to the significant radical stability and its inherent capacity to diffuse away from the site of generation, by the same logic, focal radical production by a nuclear reductase would be no more likely to generate the theoretical clustered DNA damage.

Olive (1995) reported a ratio of 10:1 for single *vs.* double strandbreaks (ssbs:dsbs) in anoxic V-79 spheroids treated with TPZ (1hr), compared with a ratio of 20:1 for X-irradiation of aerobic V79 cells. This suggests that the incidence of dsbs, arising perhaps from the random proximity of two ssbs, is not remarkably dissimilar between the two agents. The complex nature of the TPZ-induced lesions that is suggested by the very slow half-life of single-strand break repair (≈ 1hr) compared to x-rays (≈ 3-5 min) (Olive, 1995), together with the observation that TPZ-induced dsbs are protein associated (Siim *et al.*, 1996), and the possibility that TPZ itself may modify the initial DNA damage (Jones and Weinfeld, 1996), might equally imply that certain lesions are intrinsically more difficult to detect / repair. The random proximity of several lesions, or possibly a diradical-like mechanism, might suffice to produce the poorly repairable sites usually associated with the apparent "high-LET radiation-like" damage.

In a theoretical consideration of the relevance of subcellular TPZ metabolism, it is clear is that both intracellular pO_2 and pH will profoundly influence the radical half-life (Laderoute *et al.*, 1988; Wardman *et al.*, 1995; 1996). These observations, taken together with the high reported ratios of microsomal to nuclear reductive activity (Moody *et al.*, 1988), suggests multiple microenviromental factors could influence the relative contributions of the nuclear and microsomal reductase(s), with respect to the generation of the TPZ radicals that ultimately give rise to the lethal DNA damage. In the presence of moderate hypoxia it might be argued that the limited nuclear reduction of TPZ will dominate (Cahill and White, 1990; Brown, 1993), while under more severe hypoxia, the large excess in microsomal activity, together with the relative stability of the radical and its

generation in close proximity to the nucleus, could also influence the extent of DNA damage. Moreover, one must also consider the effects of high levels of microsomal O_2 consumption, that result from the very rapid metabolism and subsequent futile-cycling of the radical, as well as that arising from microsomal and nuclear-associated cytochrome P450 oxidase activity, all of which could potentially create severe microregional pO_2 gradients between the cytosolic and nuclear compartments. It has previously been suggested that increases in hypoxic toxicity could be achieved for drugs that rapidly redox cycle, and so deplete, cellular oxygen (Butler and Hoey, 1993a). O_2 consumption has been noted as a consequence of TPZ metabolism (Lloyd *et al.*, 1991), and significant cyanide-resistant respiration is seen in hepatocytes upon addition of TPZ (Silva and O'Brien, 1993). While small changes in the oxygen concentration would scarcely influence the cytotoxicity of TPZ towards aerobic cells, it would have a much greater impact on hypoxic cells. Such an effect, if it occurs at significant levels, would favourably influence the ability of microsomal-generated radicals to diffuse towards the nuclear compartment.

Chapter 4

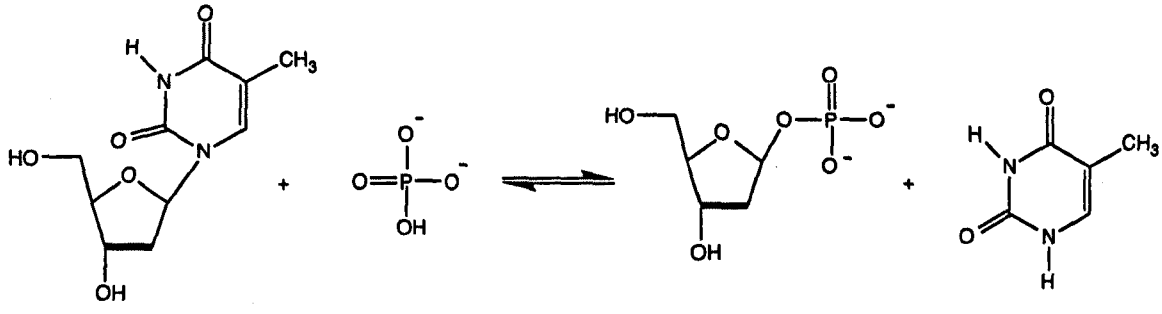
4. Evaluation of an alternative prodrug / enzyme system: Metabolic activation of 5'-deoxy-5-fluorouridine by human thymidine phosphorylase.

4.1 Introduction

An alternative approach to the development of hypoxia-specifc cytotoxins is to exploit the specific biochemical changes in hypoxic cells, or to utilise the properties of metabolic enzymes that are upregulated as a consequence of tissue hypoxia. An example of the former is the use of glucose analogues, such as 2'-deoxy-D-glucose and 5-thio-D-glucose, which exhibit selective toxicity towards hypoxic cells (Song *et al.*, 1976; 1978; Tannock, 1983) due to their preferential uptake by malignant tissues (Weber *et al.*, 1982), which is thought to arise, in part, through a dependence on high rates of anaerobic glycolysis. These glucose mimics can inhibit glucose uptake, and in their phosphorylated forms can competitively inhibit glucose utilisation (Chen and Wistler, 1957; Wick *et al.*, 1957). An example of the latter approach is the angiogenic factor platelet-derived endothelial cell growth factor (PDECGF) which is significantly induced in response to hypoxia and acidosis (Griffiths *et al.*, 1997; Takebayashi *et al.*, 1997).

Recently it was realised that PDECGF is identical to thymidine phosphorylase (Moghaddam *et al.*, 1995) being a product of the same gene. Thymidine phosphorylase (dThdPase) catalyses the reversible phosphorolytic cleavage of thymidine (dThd) and deoxyuridine to their respective bases and deoxyribose 1-phosphate, but can also metabolise fluorinated analogues in a similar fashion (Iltzsch *et al.*, 1985; Mahmoud *et al.*, 1993). This may provide an opportunity to target prodrug metabolism to tumours, since evidence strongly implicated dThdPase in the metabolic activation of the prodrug 5'-deoxy-5-fluorouridine (5'-dFUR) to the anti-neoplastic agent 5-fluorouracil (5-FU) (Fujimoto *et al.*, 1985). However at the time of this work, no publication has definitively demonstrated that human dThdPase was the

principal enzyme responsible for catalysing the phosphorolytic cleavage of the glycosidic bond of the metabolically inactive thymidine analogue 5'-dFUR, to yield the mitotic poison 5-FU.



thymidine orthophosphate ribose-1-phosphate thymine

Figure 1: Reversible phosphorolytic cleavage of the fluorinated thymidine analogue, Furtulon (5'-dFUR) to the anticancer agent, 5-fluorouracil (5-FU) and deoxyribose 1-phosphate.

5-FU is anabolically metabolised via the pyrimidine nucleoside and nucleotide pathways, mediating its toxic effects through DNA / RNA incorporation (Major *et al.*, 1982; Kufe and Major, 1981) (Figure 2). Inhibition of thymidylate synthase (TS) activity, through the formation of a ternary complex with 5,10-methyl-dihydrofolate and the 5-FU anabolite FdUMP, can also compromise *de novo* thymidine synthesis resulting in a potentially cytotoxic "thymidineless" state (Dananberg *et al.*, 1974; Santi and McHenry, 1972). This is also the primary mechanism by which the pure TS inhibitor ZD1694 exerts its cytotoxic effects (Jackman *et al.* 1991a, 1991b).

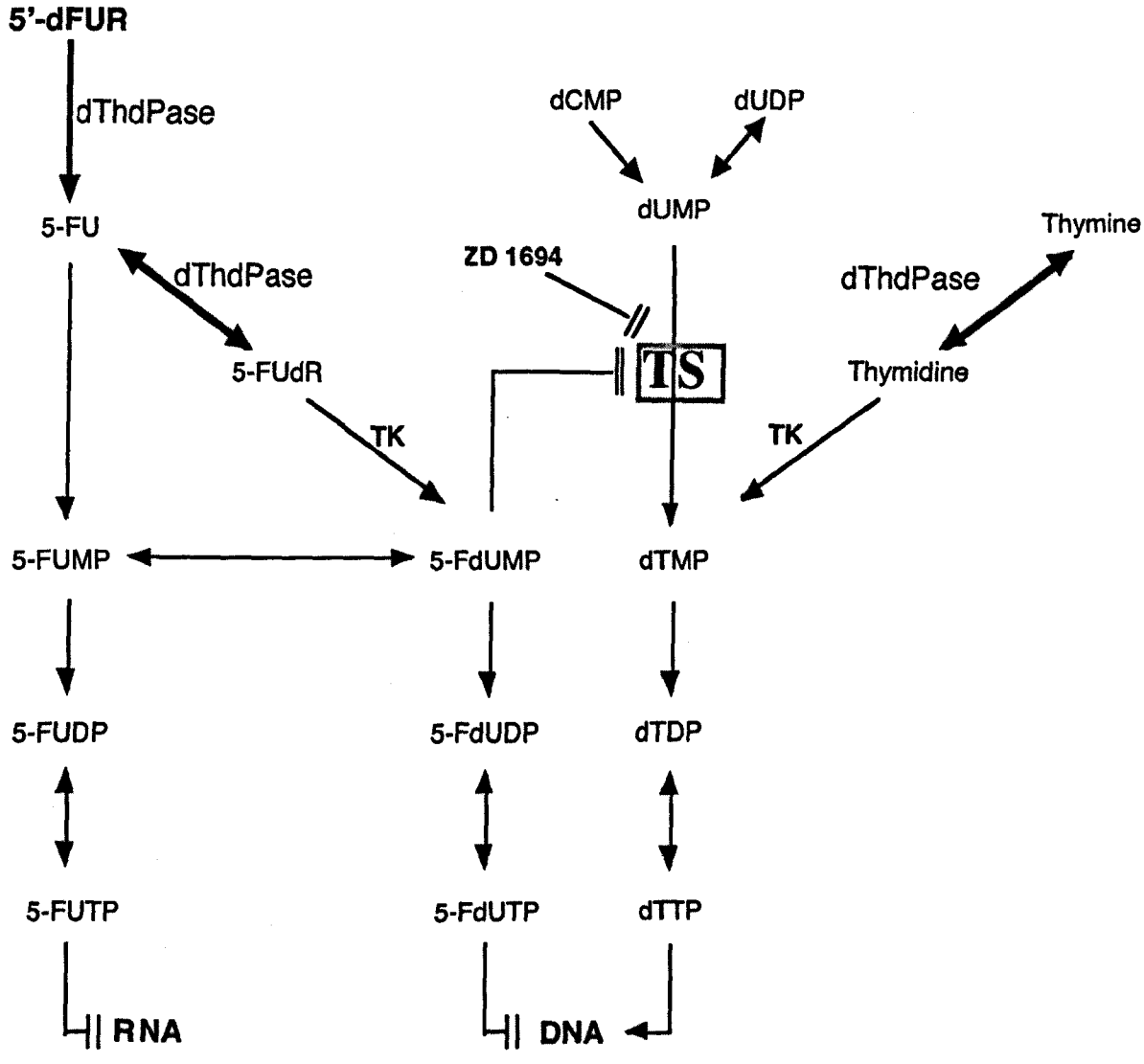


Figure 2: Schematic representation of the *de novo* and salvage pathways of thymidine and its fluorinated analogue and prodrug 5'-deoxy-5-fluorouridine (5'-dFUR).

Expression of thymidine phosphorylase (dThdPase) is markedly elevated in many solid tumors including; breast, oesophageal, pancreas, lung, bladder, ovarian, gastric and colorectal cancers, but a wide range of activities have been reported (Zimmerman and Seidenberg, 1964; Yoshimura *et al.*, 1990; Moghaddam *et al.*, 1995; O'Brien *et al.*, 1995; Takebayashi *et al.*, 1996a,

1996b; Koukourakis *et al.*, 1997; Reynolds *et al.*, 1994). This heterogeneity was examined using dThdPase enzyme activity assays of a sample group of breast tumour cytosols prepared from primary excision biopsies.

Utilising two stable clones of the human breast tumour cell line MCF-7 (TP-7 and TP-4), transfected with human dThdPase cDNA under the control of the CMV early gene promotor, the characterisation of enzyme activity with respect to dThd and 5'-dFUR metabolism and cytotoxicity was undertaken (Patterson *et al.*, 1995a).

4.2 Methods

Cell lines. The human MCF-7 adenocarcinoma breast cell line (passage 60 to 80) and clones TP-4 and TP-7 were grown in DMEM media and supplemented as described previously.

Transfection of dThdPase cDNA into MCF-7 cells. Transfection and isolation of dThdPase clones was carried out by Dr. H. Zhang at the Institute of Molecular Medicine, ICRF, Oxford. (Moghaddam *et al.*, 1995). Briefly, pCDNA3 vector containing full length dThdPase DNA was introduced into MCF-7 cells by electroporation. Stable transfectants were selected by long term incubation in 0.5 mg/ml Geneticin.

Quantification of drug sensitivity. The MTT-proliferation assay was used to determine the dose response curves of the parental and clonal cell lines as described previously. IC_{50} values were determined relative to control wells containing no drug. All incubations were 7 day aerobic exposures.

Preparation of cell lysates. Cell lines were harvested, washed and sonicated using a MSE Soniprep 150 for 3 x 5 sec (nominal frequency of 23kHz and an oscillation amplitude of 5-10 μm). Sonication was performed in 50mM Tris-HCl, 0.15M NaCl buffer, pH 7.4 at 4°C. Suspension was centrifuged at 10,000g for 15 minutes (4°C). Supernatants were stored in liquid N_2 until required.

Preparation of breast tissue cytosols. Breast tissue was removed during primary biopsy and stored in liquid N_2 until preparation. Samples were ground by pestle and mortar in the presence of liquid N_2 prior to automated homogenisation in identical buffer at 4°C. Cell debris

was removed by spinning at 3000g for 10 minutes (4°C). The resulting supernatant was spun at 100,000g for 40 minutes (4°C) and stored at -80°C.

dThdPase activity determinations. Enzyme activity determinations were conducted for parental MCF-7 and cloned TP-4 and TP-7 lines in vitro, and also for 20 breast tissue samples, 12 excision biopsies of primary neoplasia and 10 adjacent normal breast tissue samples. Lysates were incubated for 16h at 37°C in 10mM dThd or 5'-dFUR and 10mM K3PO4, pH 7.4. The reaction was terminated by addition of 0.7ml of ice cold NaOH (500mM for dThd substrate, 20mM for 5'-dFUR substrate) to 0.3ml of reaction mixture, to produce a final solution pH of 13.3 and 12.0 respectively. Quenched samples were kept on ice, and the conversion of dThd to thymine and 5'-dFUR to 5-FU was measured spectrophotometrically at 300nm and 305nm respectively (Schwartz, 1978; Choong and Lee, 1985). Optical densities were related to standard plots for known thymine and 5-FU concentrations. Breast tissue cytosols were only assayed with respect to dThd metabolism, due to limited sample availability. The protein concentrations of the cell lysates and breast tumour and normal tissue cytosols were determined using the Bio-Rad protein dye assay as described previously (Bradford, 1976). dThdPase activity is expressed as nmol substrate converted per mg total cytosolic protein per hour., and all samples were stored in liquid N_2 until required (< 6 weeks).

Influence of ZD1694 exposure on [^{3}H] dThd uptake. Subconfluent monolayers of MCF-7 parental and TP-4 cells were incubated for 4hr with or without ZD1694 (10-100 nM) in serum free media. 0.3 μM [^{3}H]dThd (20 μCi / mMol) was added for 30 min, after which the media was rapidly aspirated. Cells were washed 3 times in ice-cold calcium and magnesium free PBSA, harvested by tripsinisation and pelleted (4°C). The supernatant was discarded and 0.5 ml ice-cold 0.4 M PCA added to the pellet and mixed thoroughly. After standing on ice for 30 min, the acid insoluble material was pelleted at 1600g for 20 min (4°C). Supernatant, containing the acid soluble nucleotide pool, was spotted on silica gel 60 TLC plates, and dThd and thymine were separated in chloroform : acetone : methanol (17 : 3 : 1). The solvent front was run to the top of the TLC sheet, and spots corresponding to dThd and thymine were visualised under UV illumination (254 nm), recovered and dispensed into scintillation vials for counting. The acid insoluble pellet was washed twice with 0.2 M PCA and solubilised in 0.5 ml 0.3 M KOH prior to transfer to scintillation vials.

Western immunoblot analysis. Analysis was performed as described previously, except that the primary antibody incubation was with specific anti-human dThdPase rabbit antibody (dilution 1:500).

4.3 Results.

dThdPase determinations. The parental MCF-7 cell line had some endogenous dThdPase activity, while cell lysates of TP-7 and TP-4 displayed a 5.6 and 67 -fold elevation, respectively, in their ability to catalyse the conversion of the prodrug 5'-dFUR to 5-FU. This unequivocally implicates dThdPase in the metabolism of the prodrug 5'-dFUR. The data are summarised in table 1.

Table 1: dThdPase activities of parental and clonal cell line lysates with respect to both dThd and 5'-dFUR phosphorolytic cleavage.

	Thymidine Phosphorylase activity of cell lysates ± s.e.m. @ 37°C	
Cell line	nmol thymine released/mg/h	nmol 5-FU released/mg/h
MCF-7 wt	38.2 ± 5.9	47 ± 11.2
TP-4	3383 ± 133	3160 ±197
TP-7	269 ± 12.2	264 ± 19.4

dThdPase activity of normal and malignant breast tissue. Considerable heterogeneity was found in both the normal and tumour cytosol samples, although dThdPase activity was consistently elevated in the breast tumour cytosols ($P < 0.0002$). Values ranged from 46.5 to 929 nmol/hr/mg (median 273 nmol/hr/mg) while normal tissue cytosols displayed a more modest variability, ranging from 1.6 to 47 nmol/hr/mg (median 10.6 nmol/hr/mg). However none of the breast tumour cytosols showed elevation in dThdPase activity in the order of that found for TP-4, whilst TP-7 represents the levels at the upper 1/3 of the tumour dThdPase range (figure 3). dThdPase activity did not correlate with ER or EGFR status in either the tumour or normal tissue samples.

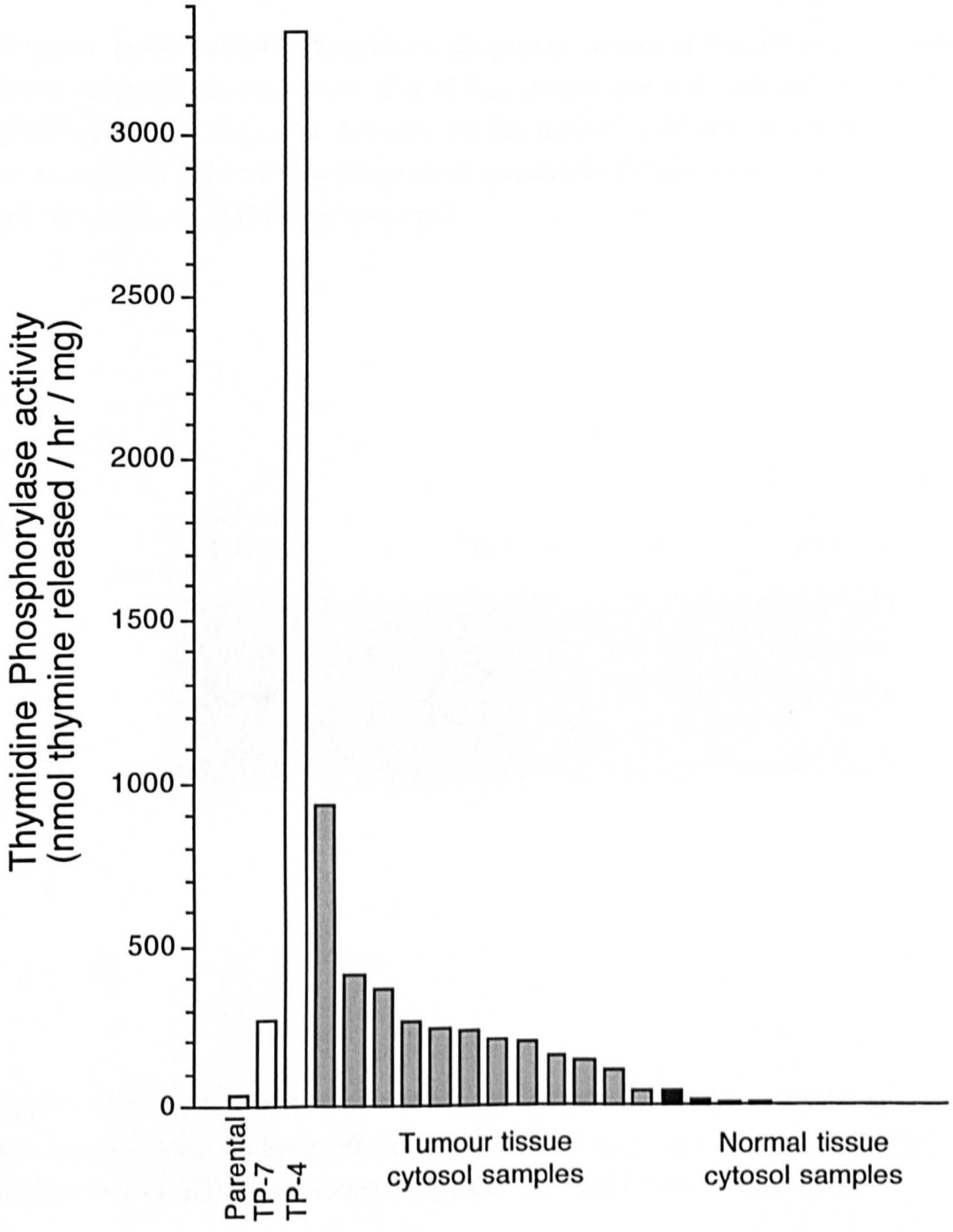


Figure 3: Relative dThdPase activity (nmol thymine released h-1mg-1 protein) of the parental MCF-7, TP-4 and TP-7 lysates relative to 12 breast tumour and ten normal breast tissue cytosols. dThdPase activity was and protein conent were determined independently at least twice and mean values are shown.

Western immunoblot. Analysis of the protein content of the cell lines confirmed that the clones expressed elevated levels of a 45 kda protein that was detected by a polyclonal anti-dThdPase antibody (figure 4). Although enzyme activity could be detected, western blotting was not as sensitive and could not demonstrate measurable dThdPase protein in the parental MCF-7 cells unless excess SDS extract was used.

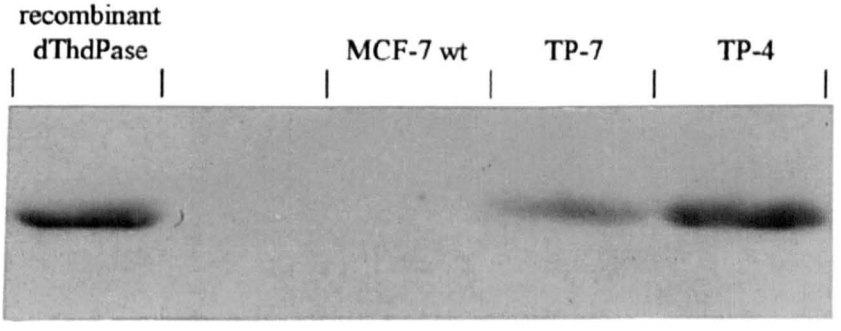
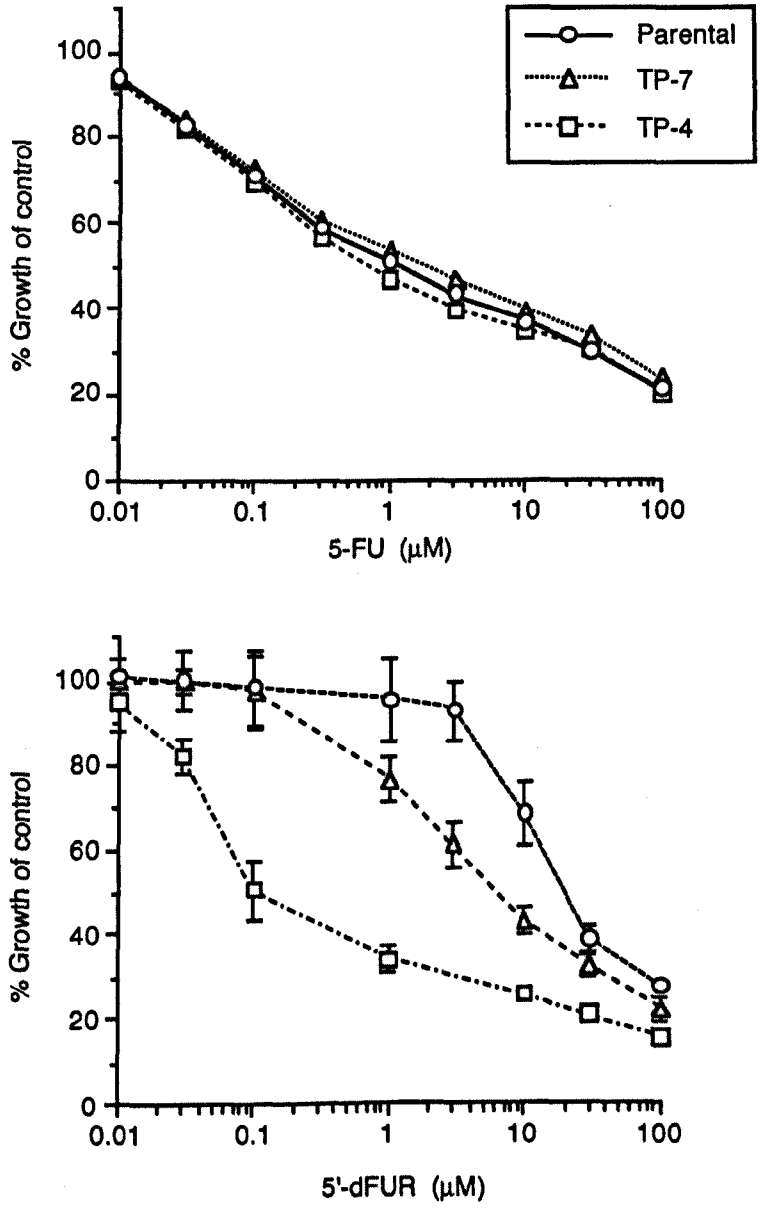


Figure 4: Western immunoblot of recombinant dThdPase , MCF-7 parental line TP-7, and TP-4 with anti-dThdPase antibody. 30 μg of protein from each cell line sample was loaded per lane, resolved in 10% SDS, and detected using ECL chemiluminesence (Amersham).

***In vitro* drug sensitivity of the cell lines.** Dose response curves were determined for 5-FU, 5'-dFUR, 5-fluoro-2'-deoxyuridine (5-FUdR). The IC_{50} values for 5-FU were not significantly different between the parental line and TP-7 and TP-4 (Figure 5). However the IC_{50} values of the prodrug 5'-dFUR were markedly different, TP-7 and TP-4 being 2.4 and 165 -fold

more sensitive as determined by IC_{50} ratios (figure 6). The differing sensitivities of the cell lines were reflected in their levels of dThdPase activity, with respect to the release of 5-FU from 5'-dFUR. Sensitivity to 5-F-2'dUR was not significantly different between the parental and clonal lines (table 2). The IC_{50} dataaresummarised in table 2.



Figures 5 & 6: Representative in vitro dose-response curves of parental MCF-7 (O), TP-4 (□) and TP-7 (Δ) to 5-FU (fig.5) and to its prodrug 5'-dFUR (fig.6). Per cent growth inhibition is relative to untreated controls. Error bars represent the s.d. of eight wells.

Table 2: IC50 values of three fluorinated pyrimidines for the parental and clonal MCF-7 cell lines.

	Drug sensitivity / IC_{50} values ± s.e.m.		
Drug	MCF-7 wt	TP-4	TP-7
5-FU (μM)	1.03 ± 1.0	0.73 ± 0.48	1.44 ± 0.96
5'-dFUR (μM)	17.3 ± 3.1	0.104 ± 0.032	7.1 ± 1.7
5-FUdR (nM)	2.3 ± 0.42	2.6 ± 0.31	2.2 ± 0.7

Sensitisation of neighbouring cells. Addition of a small fraction of TP-4 cells markedly sensitised neighbouring parental cells to the action of 5'-dFUR (figure 7). The IC_{50} of a population containing 20 : 80 mixture of TP-4 and parental cells was reduced 10-fold. This represents a significant *in vitro* "bystander" killing effect at a concentration at which the parental line is refactory to the effects of 5'-dFUR.

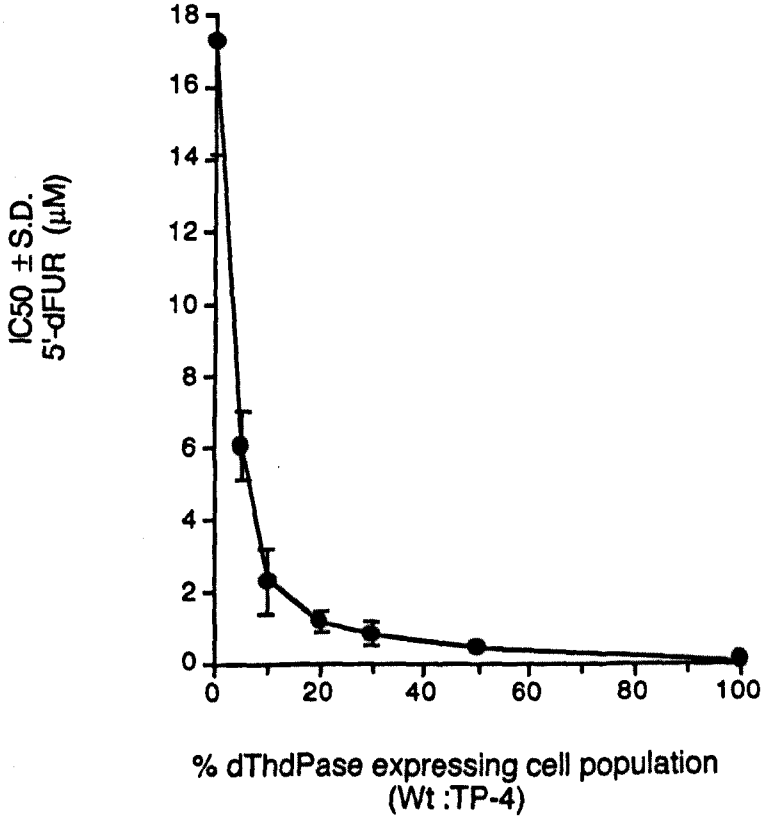


Figure 7: Plot of change in the mean IC_{50} value of the parental MCF-7 cell line for 5'-deoxy-5-fluorouridine with increasing proportions (%) of TP-4 cells, at a final cell density of $2x10^3$ /well. Cell-to-cell contact was negligible. Individual wells were exposed to a range of 5'-dFUR concentrations for 7 days before being assayed for growth relative to an untreated control, *in vitro*. IC_{50} values are the mean of at least three independent experiments ± s.e.m..

This suggests that the active anti-metabolite 5-FU, can diffuse from its site of formation and exert its effects upon neighbouring cells *in vitro*. It has been suggested that an important pathway for the bystander effect is via gap junctions (Freeman *et al.*, 1993). This is certainly true for phosphorylated metabolites (Bi *et al.*, 1993). However in the case of the MCF-7 cell line, micro-injection of the fluorescent dye Lucifer Yellow CH into single MCF-7 cells in confluent culture clearly demonstrated the absence of functional gap junctions in this cell line, ruling out the involvement of metabolic co-operation in the facilitation of the bystander effect. Since 5-FU (but not its phosphorylated anabolites) can also diffuse via a facilitated transporter, the presence of gap junctions is not essential for the sensitisation of untransfected neighbouring cells. This phenomenon may be advantageous since gap junction communication could be down-regulated in some solid tumour *in vivo* (Pitts, 1994). However, it has been observed that only relatively low levels of cell-to-cell coupling is required to permit extensive metabolite exchange (Denning and Pitts, 1997).

Since dThdPase may potentially be secreted (Kuchler, 1993), the relative contributions of intra- and extra-cellular dThdPase were assessed in two further experiments:

i. Co-incubation experiments were repeated utilising individual well inserts to spatially separate the parental and TP-4 cell lines. Anaphore membranes of 0.02, 0.2 amd 8.0 μm pore sizes were used to connect the two cell culture compartments in the media phase. Dose response curves following 5'-dFUR treatment demonstrated no difference in the ability of the TP-4 cells to sensitise the parental line to the prodrug for any of the pore sizes (data not shown). The stringent molecular cut-off imposed by the 0.02 μm anapore membrane (< 0.5 kda), or the highly porous 8.0 μm membrane (>1000 kda) did not influence the observed bystander effect in this model, suggesting that secreted or lytically released dThdPase in the extracellular media was not detectably contributing to the sensitisation of the parental line.

ii. In a further experiment, the ability of MCF-7wt or TP-4 cell line "conditioned media" to sensitise the parental cells to 5'-dFUR was assessed. Media were conditioned for 24h in the presence or absence of the prodrug and was subsequently transferred on to parental cells in exponential growth phase. In the case of media conditioned in the absence of 5'-dFUR, the prodrug was added post-conditioning. The results (table 4) clearly demonstrate that the prodrug must be co-incubated with TP-4 cells to effect a cytotoxic response in the parental line.

Table 4: Influence on media conditioning in the presence and absence of the 5'-dFUR.

	48 h CONDITIONING @ $1x10^6$ cells ml^{-1}		
DRUG	Media source	± Drug conditioning	IC_{50} values (μM)
5-FU	New	none	2.73
5-FU	TP-4 conditioned	none	2.25
5-FU	Wt conditioned	none	2.47
5'-dFUR	New	none	17.6
5'-dFUR	Wt conditioned	none	21.2
5'-dFUR	Wt conditioned	Wt conditioned	25.4
5'-dFUR	TP-4 conditioned	none	21.0
5'-dFUR	TP-4 conditioned	TP-4 conditioned	6.2 *
5'-dFUR	New	TP-4 conditioned	6.0 *

* indicates statistical significance compared to non-conditioned control ($P < 0.05$). IC_{50} values are the mean of two independent experiments.

Co-incubation of 5'-dFUR with the parental line did not result in a cytotoxic response. Furthermore, pre-conditioning of the media in the absence of 5'-dFUR, followed by its post-addition, was not sufficient to elicit the subsequent metabolic release of the prodrug. Analysis of the relative proportions of dThdPase protein in the intra- and extracellular fractions following 24h media conditioning was conducted by western blotting (figure 8). Visualisation of total dThdPase protein in cell extracts, versus conditioned media concentrated 100-fold by Macrostep centrifugal concentration (10 kda cut-off), confirmed the presence of at least a 1000-fold excess of intracellular dThdPase protein.

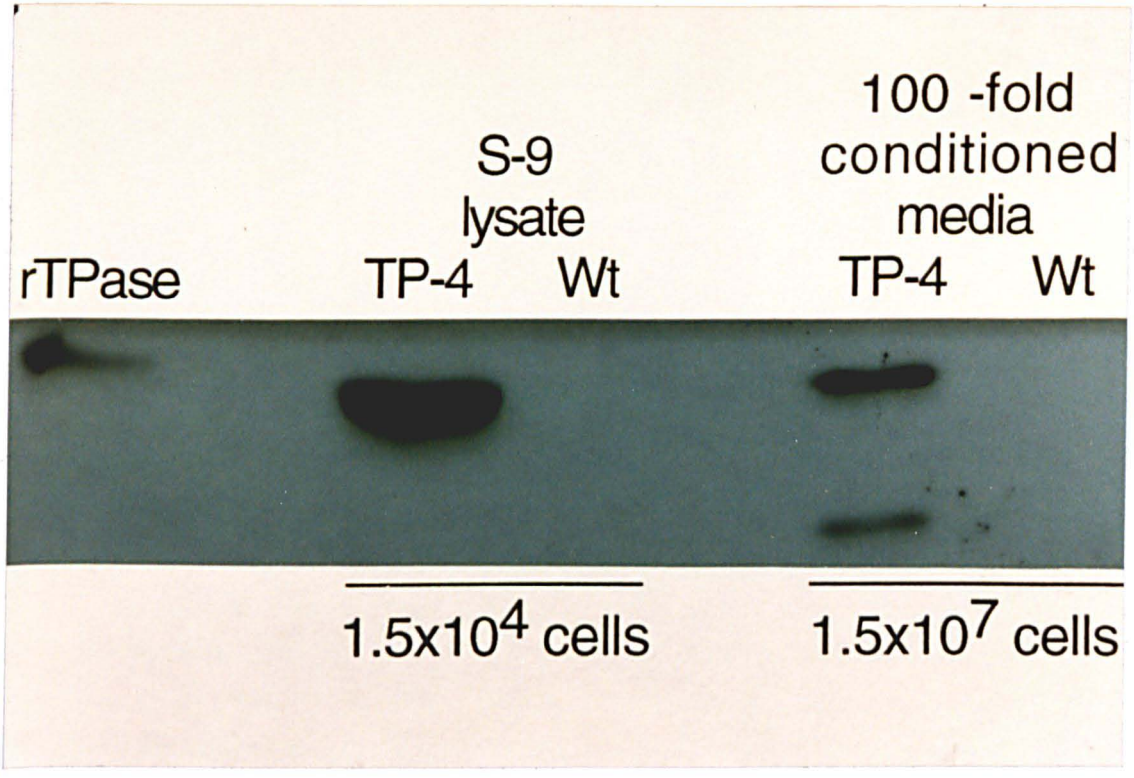


Figure 8: Analysis of the relative proportions of dThdPase protein in the intra-and extra-cellular fractions following 24h media conditioning was conducted by western blotting of MCF-7 wt and TP-4 cell lysates along side conditioned media. dThdPase protein in conditioned media was concentrated up 100-fold by Macrostep centrifugal concentration (10 kda cut-off). Densitometric analysis confirmed the presence of at least a 5000-fold excess of intracellular dThdPase protein in the TP-4 cell line.

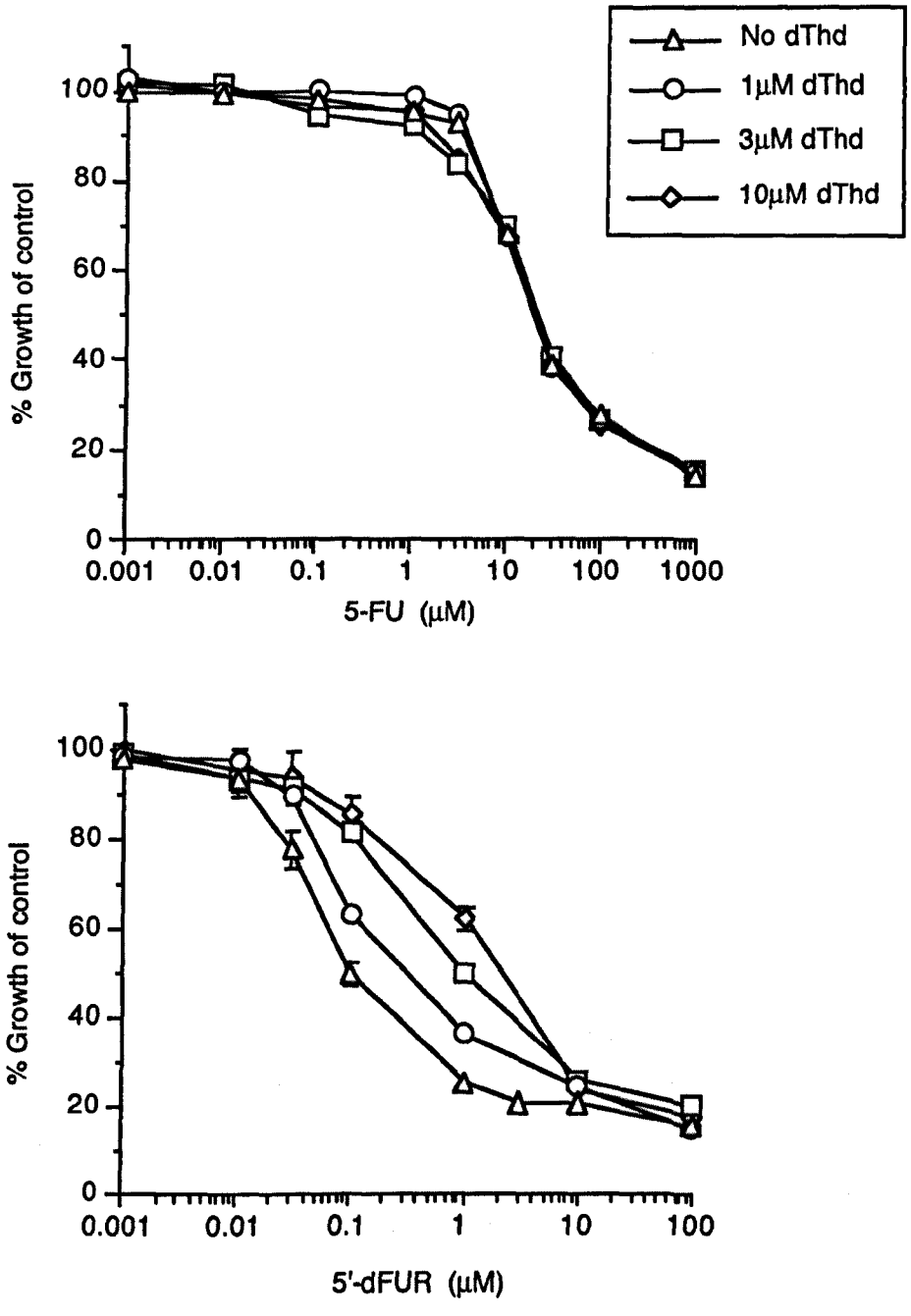
Modulation of drug sensitivity by exogenous thymidine. Circulating dThd is present in the plasma of individuals at 0.1 - 0.2 μM (Shaw *et al.*, 1988a, 1988b). While the extent of vascularisation of a solid tumour largely dictates the bioavailability of such nutrients, dThd availability in the microenvironmentof a tumour may become elevated as a result of release from dying cells. Such increased bioavailability of dThd could modulate the efficacy of the prodrug 5'-

dFUR by competing for the phosphorolytic action of ThdPase. The cytotoxic effects of 5-FU are thought to be mediated, in part, by the inhibition of TS, through the anabolism of 5-FU to 5-FdUMP (Dananberg *et al.*, 1974; Santi and McHenry, 1972). Therefore the presence of salvageable dThd may also circumvent any toxicity associated with the inhibition of TS activity. Consequently, the capacity of physiologically relevant concentrations of dThd to modulate the toxicity of 5'-dFUR, 5-FU and 5-FUdR was determined *in vitro*.

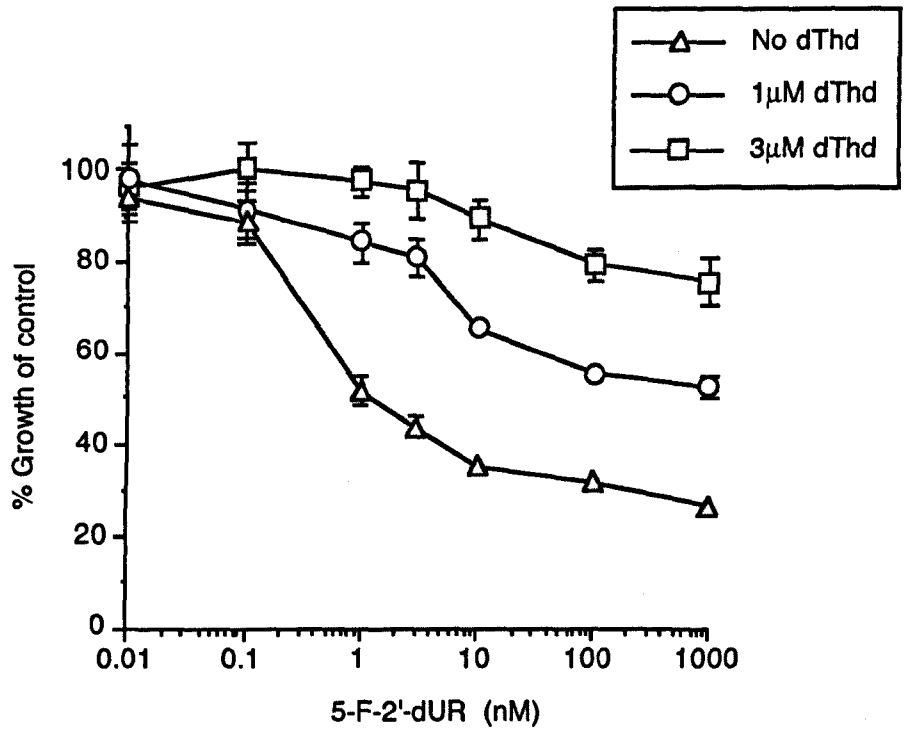
Co-addition of dThd during 5-FU exposure did not affect the sensitivity of either the parental or transfected cell lines, even at the maximum concentration (150 μM) that was nontoxic to the cells (data not shown). This suggests that TS inhibition is not an important determinant for 5-FU toxicity in the MCF-7 cell line, since inhibition of *de novo* thymidine synthesis can be circumvented through the availability of an exogenous dThd source which can be salvaged via facilitated transport.

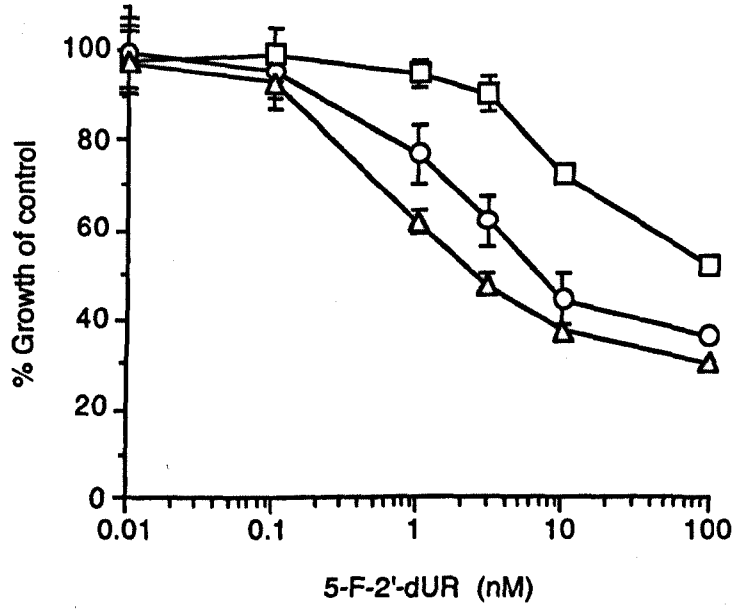
1-10 μM dThd had no effect on the response of the parental line to 5'-dFUR (figure 9). In contrast, physiologically relevant concentrations of dThd (1-10 μM) could partially reverse the inhibitory activity of 5'-dFUR on TP-4 and TP-7. Indeed, 10μM dThd shifted the IC_{50} value of TP-4 for 5'-dFUR by 18-fold (figure 10). Nevertheless, TP-4 cells were still markedly sensitised to 5'-dFUR compared to controls, and sufficiently high prodrug concentrations (≥10μM) could overcome the dThd-induced reversal of toxicity. Thus even levels of dThd 50-100 fold greater than those detectable in plasma could not fully reverse the cytotoxicity of 5'-dFUR in this breast cell line model *in vitro*.

There was a marked capacity of exogenously added dThd (1-3 μM) to modulate the inhibitory effects of 5-F-2'dUR in the parental cell line, which was significantly reversed in the clone cells, particularly TP-4. This suggests that the phosphorolytic breakdown of dThd by dThdPase renders it metabolically unavailable to bypass the inhibition of thymidylate synthase, or to compete with 5FdUTP for incorporation into DNA (Nayak, 1992). Indeed, 1 μM dThd could increase the IC_{50} value of 5-F-2'dUR for the parental line from 2.3 to ~ 2400 nM, some 1000-fold (figure 11), while producing only a 3-fold reversal of toxicity in TP-4, from 2.6 to 8.0 nM (figure 12).



Figures 9 & 10: Representative in vitro dose-response curves of the parental MCF-7 cell line (fig.9) and the dThdPase transfected TP-4 cell line (fig.10) to 5'-dFUR, in the absence (Δ) or presence of exogenously added thymidine, at a concentration of 1 μM (O), 3 μM (□) and 10 μM (◊).

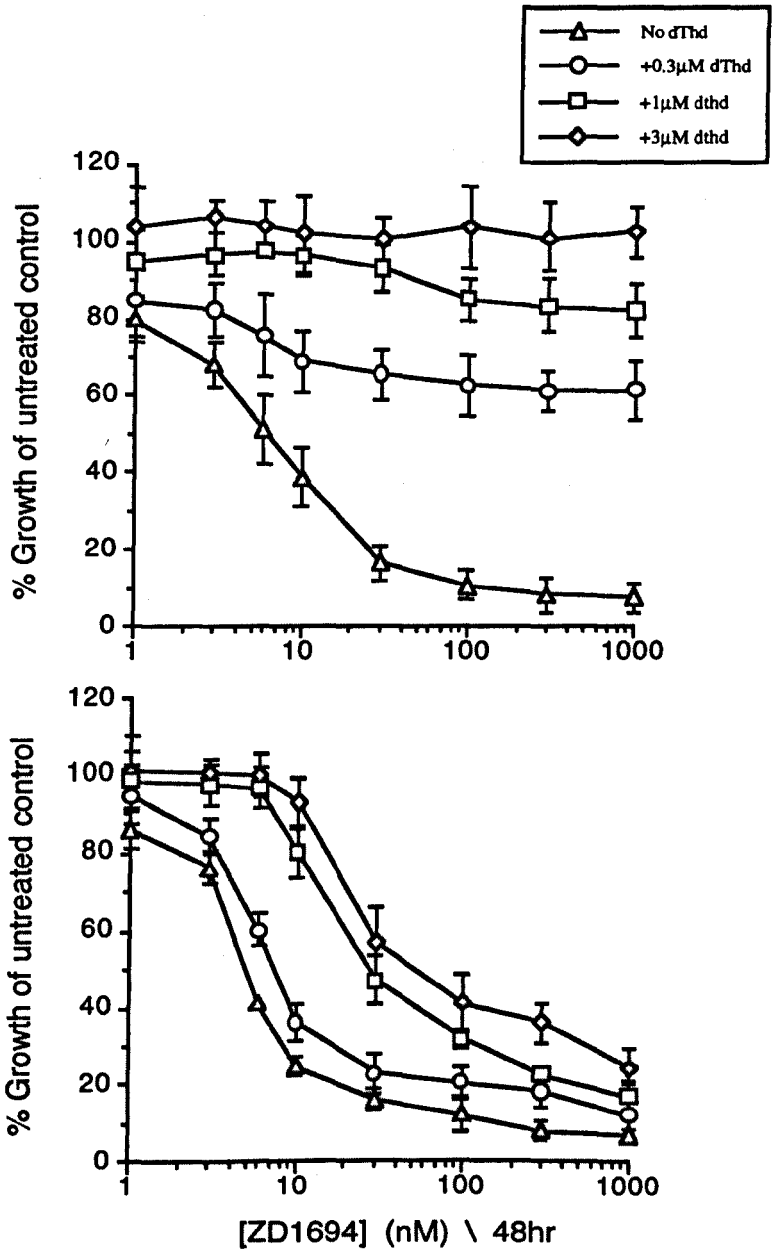




Figures 11 & 12: Representative in vitro dose-response curves of the parental MCF-7 cell line (fig.11) and the dThdPase transfected TP-4 cell line (fig.12) to 5F-2'-dUR, in the absence (Δ) or presence of exogenously added thymidine, at a concentration of 1 μM (O) and 3 μM (□).

In order to study the role of dThdPase in modulating the ability of the MCF-7 cells to salvage dThd following TS inhibition, ZD1694 was utilised. ZD1694 is a quinazoline antifolate thymidylate synthase inhibitor that is extensively polyglutamated by folylpolyglutamate synthase resulting in enhanced intracellular retention (Jackman *et al.*, 1991a, 1991b). Since ZD1694 selectively inhibits TS activity upon polyglutamation, addition of exogenous dThd should be sufficient to prevent cytotoxicity. In the absence of salvagable extra-cellular dThd, a "thymidineless" state arises, and the concurrent perturbationsof the nucleotide triphosphatepools (Dananberg *et al.*, 1974; Santi and McHenry, 1972) are considered the dominant mechanism by which ZD1694 exerts its cytotoxic effects. dThdPase is the first catabolic enzyme in the dThd salvage pathway, and thus represents a potential mechanism by which dThd can be rendered unavailable for subsequent phosphorylation by thymidine kinase (TK). While the phosphorolytic cleavage of dThd and deoxyuridine to their respective bases and deoxyribose 1-phosphate (Iltzsch *et al.*, 1985; Mahmoud *et al.*, 1993) are reversible reactions, with an equilibrium constant close to one, the limited availability of deoxyribose donors favours the phosphorolytic over the transferase reaction. The utilisation of thymine in cells is generally less than 1% of dThd incorporation; supporting the evidence that dThd catabolism dominates thymine anabolism (Gallo and Breitman, 1968; Iltzsch *et al.*, 1985; Schwartz *et al.*, 1994).

The addition of exogenous dThd (1-3μM) for 96 h, during and following a 48 h drug exposure, could almost completely reverse the cytotoxicity resulting from TS inhibition in the parental cell line (figure 13). However, experiments conducted using TP-4 demonstrated that the presence of elevated dThdPase activity could very significantly modulate this "thymidine rescue" phenomenon (figure 14). Addition of 0.3μM dThd (96hr) resulted in a significant modulation of the dose-response curve for the parental line, such that an IC_{50} value was no longer determinable (fig.13). In contrast 0.3μM dThd rescue in the TP-4 cell line only shifted the IC_{50} value from 3.5 to 8.0 nm (fig.14). Increasing concentrations of exogenous dThd (1.0 and 3.0 μM) were more effective at reversing the ZD1694-dependent toxicity in both cell lines. However the rescue was far superior in the parental line. Indeed, no significant inhibition of growth could be achieved in the parental line at either 1.0 or 3.0 μM dThd, while IC_{50} values for the TP-4 line were 26 and 88 nm respectively. This represents a very significant modulation of rescue at physiologically relevant dThd concentrations and suggests that, within this *in vitro* model, elevated dThdPase activity need only be present during the initial period of TS inhibition inorder to compromise any dThd-dependent rescue phenomena. (Patterson *et al.*, 1998).



Figures 13 & 14: Representative dose-response curves for parental MCF-7 (fig. 13) and TP-4 (Fig. 14) cell lines to 48 h ZD1694 treatment. Exogenous thymidine was absent (Δ) or present during and for a further 48 h post-ZD1694 exposure, at concentrations of 0.3 μM (O), 1.0 μM (□) and 3.0 μM (◊). Cells were allowed to proliferate for a further 8 days following removal of dThd (total growth time = 12 days). Per cent growth inhibition was expressed relative to untreated control growth.

Uptake and metabolism of [^{3}H]-thymidine in ZD1964 treated MCF-7 cells. Following a 4h treatment at 3 or 30 nM ZD1694, parental and TP-4 cells were pulsed with 30 mins. 0.3μM [^{3}H] thymidine, prior to harvesting and acid extraction. Total uptake of [^{3}H]-label was similar in both cell lines under comparable conditions. Consistent with the impact of exogenous dThd on the modulation of the dose-response curves of the parental, but not the TP-4 cell line, a greater proportion of the initial thymidine up take was metabolised to thymine (figure 15). At 30nM ZD1964 pre-treatment, 27% of the total salvaged dThd was rendered metabolically unavailable to relieve the acute TS inhibition in the TP-4 cells, combared to just 3.6% in the parental cell line. Therefore the presence of intracellular dThdPase activity could modify the extent to which dThd was available via the nucleotide salvage pathway.

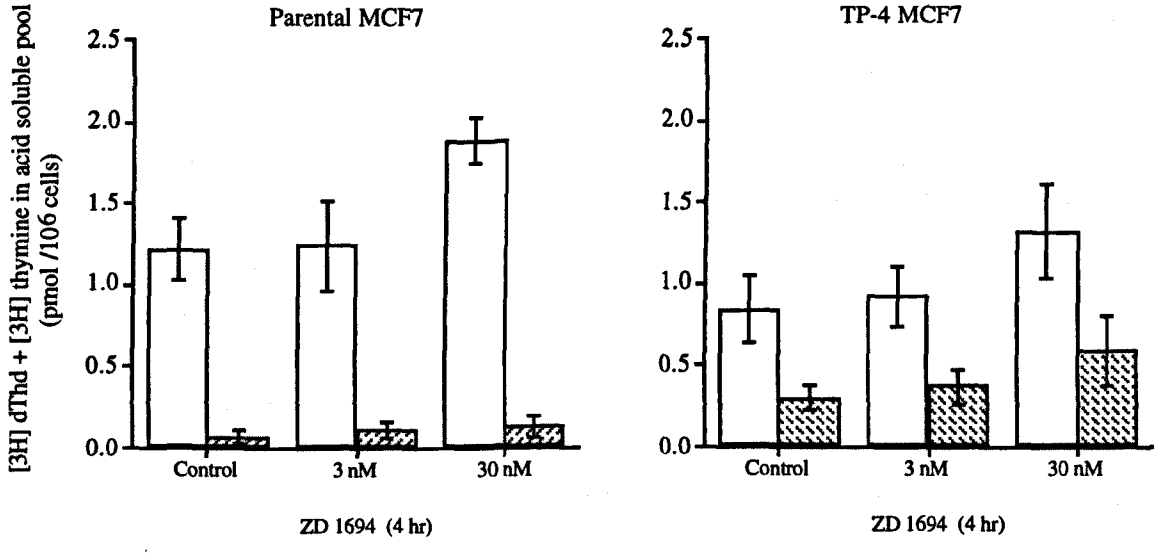


Figure 15. Influence of ZD1694 exposure on [^{3}H] dThd uptake in MCF-7 parental and TP-4 cells. Cultures were incubated for 4hr with or without ZD1694 (3 and 30 nM) in serum free media. 0.3 μM [^{3}H] dThd (20 μCi / mMol) was added for 30 min, after which the media was rapidly aspirated. Cells were harvested (4°C) and [^{3}H] dThd (open bars) and [^{3}H] thymine (hatched bars) were separated by TLC and counted.

4.4 Discussion

Introduction of the gene encoding human dThdPase, sensitised the human breast cancer cell line, MCF-7 to the prodrug 5'-dFUR. This provided a means of improving the chemotherapeutic selectivity of the fluorinated anti-metabolite 5-FU. Gene insertion into a small proportion of MCF-7 cells was sufficient to cross-sensitise neighbouring non-transfected cells *in vitro*, even though no functional-gap junctions could be found. Taken together, these data suggest that the targeting of a tumour mass with a tissue-specific promotor driven dThdPase sequence *in vivo*, may not require the transduction of the majority of tumour cells for effective killing of neighbouring cells to occur. Furthermore modulation of gap junction expression should not significantly compromise the efficacy of the bystander effect (Pitts, 1994; Denning and Pitts, 1997).

It is plausible that mechanistic advantages of the dThdPase / 5'-dFUR may result from the co-metabolism of endogenous dThd. In spite of being in direct substrate competition with 5'-dFUR if present at 10 to 100 -fold excess of plasma concentrations (1-10 μM), it might nevertheless enhance the cytotoxicity of 5-FU in a number of ways. Phosphorolytic cleavage of dThd by dThdPase would render it metabolically unavailable to bypass the inhibition of *de novo* dThd synthesis and to compete with FdUTP for incorporation into DNA (Major *et al.*, 1982). The "thymidine-less" state, resulting from the inhibition of TS by FdUMP, would make tissues expressing dThdPase sensitive to the depletion of salvageable dThd, restricting any potential "rescue" from the thymidine-less induced stress and its associated cytotoxicity (Houghton *et al.*, 1993). Unfortunately the MCF-7 breast cell line appeared to be atypical. This was demonstrated by the inability of exogenous dThd to rescue from 5-FU cytotoxicty and suggests that TS inhibition by the 5-FU anabolite, FdUMP, did not contribute to cytotoxicity. This observation is in agreement with other published data indicating that the primary determinant of 5-FU toxicity in the MCF-7 cell line is RNA incorporation (Kufe and Major, 1981). However the underlying principle of such a role for dThdPase could be demonstrated in the MCF-7 cell line, through the use of the specific TS inhibitor ZD1694.

dThdPase can also catalyse the metabolism of 5-FU itself, facilitating the formation of FdUMP through the reversible addition of deoxyribose-1-phosphate. Thus it would be anticipated that this subsequent anabolism of the prodrug-released 5-FU by dThdPase, could result in 5-FU ultimately proceeding via the significantly more cytotoxic nucleotide pathway. This could lead to

increased DNA incorporation, particularly if synthetic ribose-1-phosphate donors were also present during 5-FU anabolism (Schwartz *et al.*,1994).

Depletion of the available dThd would also serve to increase local concentrations of thymine which would competitively inhibit the catabolism of 5-FU by dihydrouracil dehydrogenase, potentially extending its half-life within the tumour mass (Santelli and Valeriote, 1980). Prolonging the duration and intensity of tumour tissue exposure to 5-FU, and its associated anabolites, has been shown to limit the occurrence of resistant clones associated with sub-optimal chronic exposures *in vitro* (Sobrero *et al.*, 1993). This may have implications in restricting the development of acquired resistance in vivo. Increasing the duration of 5-FU exposure has also beeen shown to significantly enhance the cytotoxicity of the bio-modulators leucovorin and interferon-α2A *in vitro* (Houghton *et al.*, 1993).

It is possible that the generation of 5-FU through the dThdPase / 5'-dFUR system as opposed to CDase / 5-FC might prove therapeutically superior, but the gain may not overcome the intrinsic problems associated with the treatment of hypoxic sub-populations within solid tumours. Nevertheless, poor cellular uptake of 5-FC has been demonstrated to be a limiting factor in the efficacy of the CDase / 5-FC system (Haberkorn *et al.*, 1996), a phenomenonthat is not encountered by the thymidine analogue, 5'-dFUR, since active pyrimidine nucleotide salvage in tumour cells often exceed that of normal tissues (Kinsella *et al.*, 1997). If utilised in combinational chemotherapy using an enzyme-directed approach, the increased tumour specificity of prodrug activation in high dThdPase expressing neoplastic tissues, may provide 5'-dFUR with superior efficacy than that documented for 5-FU (Scheithauer and Raderer 1995). Alternatively, the mechanistic advantages of the dThdPase / 5'-dFUR model could be applied in a GDEPT strategy in conjuction with a tissue-specific promoter, or in an antibody-directed approach, where a greater proportion of the cycling tumour mass could be targeted. Further, the capacity of dThdPase to compromise the ability of cells to salvage exogenous thymdine when present either intra- or extracellularly also makes it an attractive candidate for antibody-directed enzyme based therapeutic strategies in conjunction with TS inhibitors. However, the expression of dThdPase in many normal tissues may limit the tumour-specificity of this enzyme/prodrug paradigm (Fox *et al.*, 1995).

The observation that ZD1694 toxicity is totally prevented by the presence of salvageable dThd, is consistent with the primary mode of action being TS inhibition, resulting in acute inhibition of *de novo* thymidine synthesis. Critically, nucleosides are present in human plasma at

concentrations sufficient to fulfill a salvage function, and preformed dThd is potentially bioavailable within a tumour mass, both from the vascular supply and that released by dying cells. The high levels of TK in tumour compared to normal tissues (Weber, 1983) implies that the salvage pathway is a preferred source of dThd for subsequent anabolism and DNA synthesis. Cellular uptake readily occurs via S-(*p*-nitrobenzyl)-6-thioinosine -sensitive and/or -insensitive cell-surface equilibrated nucleoside transporters (Belt, 1983; Belt *et al.*, 1993), a Na^+-associated concentrative transport process (Crawford *et al.*, 1990; Griffith and Jarvis, 1993) or through non-facilitated diffusion. It has been demonstrated that nucleoside transport (NT) activity can be upregulated 22 to 39 -fold as a consequence of acute TS inhibition in the human bladder cancer cell line MGH-U1 (Pressacco *et al.*, 1995). Such elevations in NT function may be highly relevant where dThd availability is the potentially rate limiting step in the salvage pathway. Thus facilitated and non-facilitated uptake processes could provide an effective intracellular source of dThd for the salvage pathway, which could prove sufficient to circumvent the cytotoxic impact of ZD1694-mediated TS inhibition. Taken together with evidence that the contribution of salvage flux is greater than that of *de novo* synthesis in neoplastic tissues, and further, inhibition of *de novo* synthesis leads to even higher salvage activity, any approach that modulates the availability of exogenous pyrimidine nucleotides should significantly enhance ZD1694 toxicity. Consistent with this proposal, it was demonstrated that the presence of elevated dThdPase activity, the first enzyme in the thymidine salvage pathway, can markedly compromise the intracellular bioavailability of salvaged dThd.

Approaches aimed at rational patient selection based upon "enzyme-profiling" of tumor tissues could target those individuals most likely to benefit from treatment with 5'-dFUR or ZD1694. Several studies have indicated that high dThdPase expression is associated with poor prognosis (Takebayashi *et al.*, 1996b; Koukourakis *et al.*, 1997). However it has recently shown that patients treated with adjuvant cyclophosphamide, methotrexate and 5-FU for breast cancer, had a better prognosis if dThdPase was elevated in their tumors (Fox *et al.*, 1997). Both methotrexate and 5-FU would be potentiated by preventing dThd-rescue and so provides *in vivo* clinical evidence of the potential for manipulation of the salvage pathway. Thus, perhaps therapies aimed at exploiting this clinically unfavourable tumor characteristic to advantage may prove to be of value. Alternatively, approaches aimed at inducing tumour dThdPase activity, such as interferon-α and -γ treatment (Schwartz *et al.*, 1994), may enhance the response to 5'-dFUR and ZD1694 treatment.

Chapter 5

5. Evaluation of the hypoxia-responsive enhancer from the murine phosphoglycerate kinase-1 gene in the transcriptional regulation of gene expression.

5.1 Introduction

A range of promoter/enhancer sequences have been characterised for application in gene therapy, potentially allowing the tightly regulated expression of therapeutic genes within neoplastic tissues. Targeted gene expression can be achieved using tissue- or disease-specific promoters (Reviews: Hart, 1996; Dachs *et al.*, 1998), or alternatively through exploiting transcriptional elements that are responsive to physiological conditions unique to solid tumour microenviroments, including glucose-deprivation and hypoxia (Little *et al.*, 1994; Gazit *et al.*, 1995; Dachs *et al.*, 1997). Gene expression could also be regulated by 'coupling' transcriptional activity to externally delivered stimuli, such as ionizing radiation (Weichselbaum *et al.*, 1994; Seung *et al.*, 1995; Hallahan *et al.*, 1995).

Attentions have also focused on the unique features of tumour physiology which arise as a consequence of inefficient blood supply, and how such differences might be exploited (Brown and Giaccia, 1994; Stratford *et al.*, 1994; Dachs and Stratford, 1996). The most conspicuous of these features is tumour hypoxia. The recent observations that hypoxia responsive elements (HREs), found within a number of genes (Firth *et al.*, 1994), can specifically regulate transcription in response to biological hypoxia, has suggested that HRE-dependent expression of therapeutic enzymes might be one approach to exploiting a unique characteristic of tumour physiology. Such a HRE-driven GDEPT strategy could be applied to a broad range of solid tumours of variable tissue origin and histology, and might fulfil an important therapeutic goal.

5.1.1 The molecular basis of hypoxia responsive element dependent transcriptional regulation.

Hypoxia enhancer regions have been found in a number of genes including erythropoietin (Epo) (Goldberg *et al.*, 1987), vascular endothelial growth factor (Goldberg *et al.*, 1994), and a number of proteins involved in glucose metabolism, including; phosphoglycerate

kinase-1, aldolase A, enolase 1, lactate dehydrogenase A, pyruvate kinase M, phosphofructokinase L and glucose transporter-1 (Firth *et al.*, 1994; Semenza *et al.*, 1994; Ebert *et al.*, 1995a, 1995b; Semenza *et al.*, 1996). Among the genes encoding glycolytic enzymes, regulation by hypoxia is isozyme specific in a way that correlates with their increased expression in malignant cells (Ebert *et al.*, 1995). For example, lactate dehydrogenase (LDH) is a tetrameric enzyme with five isoforms which are composed of combinations of two subunits, A and B. The LDH-B subunit kinetically favours the conversion of lactate to pyruvate and is found at high levels in aerobic tissues such as the heart, while LDH-A converts pyruvate to lactate under nonequilibrium conditions. Only the LDH A subunit is selectively induced by hypoxia (Firth *et al.*, 1995).

The Epo gene HRE was the first enhancer to be characterised in detail. A minimal 24 base pair (bp) sequence 3' of the coding region has been defined as sufficient for hypoxia response ($\leq$ 1% O_2) (Madan and Curtin, 1993). The EPO-derived hypoxia response element (HRE) was subsequently reported to confer O_2-dependent transcription, independent of orientation, distance, homologous or heterologous promoter context and was universally functional in mammalian cells (Maxwell *et al.*, 1993). The nuclear transcription factor, Hypoxia Inducible Factor 1 (HIF-1), was shown to specifically interact with the HRE of Epo (Wang and Semenza, 1993a). Mutations of the Epo enhancer that prevented HIF-1 binding also eliminated the hypoxic induction of Epo reporter genes. Further studies demonstrated that hypoxia-mediated induction of transcription could be improved by reducing the distance between enhancer and promoter, and increased further by inserting multiple copies of the 24 bp sequence (Pugh *et al.*, 1994). The finding that hypoxically inducible activity of the Epo HRE was widespread in mammalian cells implied that an oxygen sensing mechanism was present in non-Epo producing cells, and suggested that HIF-1 was a universal transcription factor that served to regulate other oxygen-dependent genes.

5.1.2 Hypoxia Inducible Factor 1

HIF-1 is a 120 kda heterodimer found in both Epo-producing and non-producing mammalian cells (Beck *et al.*, 1993), which is upregulated and has improved DNA binding capacity as a consequence of hypoxic stress. HIF-1α/β is a heterodimeric member of a subfamily of basic-helix-loop-helix (bHLH) transcription factors defined by a conserved PAS domain. The defining members of the PAS (Per-ARNT-Sim) groups of transcription factors were the period (Per) and single-minded (Sim) proteins of *Drosophila*

melanogaster, involved in circadian regulation of gene expression and neurogenesis, respectively (Review: Littlewood and Evan, 1995). Helix-loop-helix protein structures are a highly conserved motif employed by a family of transcription factors which typically bind as dimers and recognise an approximately symmetrical site. The two α-helicies govern interaction with DNA, one by binding in the major groove of B-form DNA and the other by contacting the DNA backbone.

The 826 amino acid polypeptide HIF-1α subunit is unique to the HIF-1 heterodimer (Wang *et al.*, 1995b), while HIF-1β is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT) protein, and exists as two alternately spliced isoforms of 774 and 789 amino acids (Hoffman *et al.*, 1991). ARNT is also the heterodimeric partner for the aryl hydrocarbon receptor (AHR), which functions as a ligand-activated transcription factor that mediates the xenobiotic (polyaromatic hydrocarbon eg. dioxin) response of a number of metabolic genes (Review: Hankinson, 1995). Thus, ARNT is an essential dimerisation partner in both AHR- and HIF-1α -mediated signalling pathways, and the formation of selective complexes allows the specific recognition of either xenobiotic responsive elements (XREs) or hypoxia responsive elements (HREs), respectively. Consistent with these observations, the hypoxia and dioxin signal transduction pathways have been demonstrated to functionally compete for recruitment of ARNT (Gradin *et al.*,1996; Gassmann *et al.*, 1997), and cells functionally deficient in the xenobiotic response, due to an ARNT mutation, are also defective in HIF-1 mediated functions (Wood *et al.*, 1996). AHR and HIF-1α share some apparent similarities, such as being associated with the molecular chaperone, HSP-90, in the pre-activated state, which is probably a reflection of their common evolutionary past.

A major control of HIF-1 activity by oxygen tension is achieved through changes in the level of the HIF-1α subunit which complexes with the constitutively expressed HIF-1β/ARNT subunit, that is considered to be present in excess (Reviews: Poyton and Bunn, 1996; Semenza, 1996; O'Rourke *et al.*, 1997; Gassmann and Wenger, 1997). Dimerisation of HIF-1 is essential, but not sufficient, to stimulate the *trans*-activation functions of HIF-1. The induction of HIF-1 and Epo mRNA is ablated by inhibitors of transcription (actinomycin D) or protein synthesis (cycloheximide) demonstrating that both *de novo* transcription and protein synthesis are required in the activation of the HIF-1 dependent signal pathway (Wang and Semenza, 1993b; Wang *et al.*, 1995d; Salceda *et al.*, 1997). Hypoxic gene induction is also sensitive to protein kinase inhibitors, and evidence indicating that HIF-1 is a phosphoprotein (Wang *et al.*, 1995d) lend support for the participation of protein phosphorylation in HIF-1 *trans*-activation. This suggests a possible

cascade of protein phosphorylation and dephosphorylation in transducing the hypoxic signal from sensor to HIF-1, and is thought to be mediated in part by protein tyrosine kinases. These include the proto-oncogenes c-Src and H-Ras (Jiang *et al.*, 1997a; Mazure *et al.*, 1996; Arbiser *et al.*, 1997), although the relevance of c-Src has been questioned (Gleadle and Ratcliffe, 1995a). Signal transduction may involve the mitogen activated protein kinases, MEK1 and MEK2, perhaps mediated via PKC (Salceda *et al.*, 1997). The PKA pathway is also known to contribute to the amplitude of HRE-dependent transcription, although cAMP is not an essential component of the signal transduction pathway *per se* (Kvietikova *et al.*, 1995; 1997). cAMP enhancer binding protein-1 (CREB-1) is implicated in the integration of the hypoxia and cAMP signalling pathways. The modulation of HIF-1 activity by changes in cellular redox further suggests that signal transduction is integrated at multiple levels, and the participation of HAP-1 and TDX suggest the presence of a specific redox signal cascade (Wang *et al.*, 1995c; Huang *et al.*, 1996). Perhaps analogous, another bHLH transcription factor, USF, is functionally regulated *via* two cysteine residues in the HLH dimer interface. These critical redox-sensitive sites must be reduced to enable DNA binding and transcriptional activation (Pognonec *et al.*, 1992). Some evidence also exists to suggest that HIF-1 is involved in the cellular responsive to glucose deprivation (Maltepe *et al.*, 1997), although recent evidence has implicated the involvement of an as yet unidentified transcription factor (Woods *et al.*, 1998).

The HIF-1α subunit contains two minimal domains (amino acids 549-582, and 775-826) each of which can act independently to convey a hypoxia-inducible transcriptional response (Pugh *et al.*, 1997). Evidence suggests a dual mechanism of activation involving an inducible activation domain that is amplified by regulation of HIF-1α abundance, probably *via* protein stabilisation (Wenger *et al.*, 1997; Salceda and Caro, 1997). Thus the regulation of HIF-1α levels is integrated with changes in DNA binding activity and post-translational enhancement of activation. This is reflected in the rapid kinetics of HIF-1 association and dissociation with its DNA recognition sites, with a $t_{1/2}$ for both of less than 1 minute, while the HIF-1α subunit is readily degraded (< 5 min.) upon reintroduction of cells to normoxia. The increases in HIF-1α protein levels may be partially independent of the process of transactivation (Pugh *et al.*, 1997), although the relationship between these properties and hypoxia signal integration is not currently known. Functional *trans*-activation can also involve the recruitment of accessory co-activators. An interaction between the C-terminal portion of HIF-1α and the co-activator p300 has been demonstrated, and over-expression of p300 could amplify the transcriptional response to hypoxia (Arany *et al.*, 1996). P300, which is closely homologous to CREB protein, is a

general transcriptional co-activator that participates in multiple biological functions including induction of various tissue-specific enhancers, regulation of cell cycle, and stimulation of differentiation pathways. While p300 does not directly bind DNA, it plays a critical role in transducing the signal from the HIF-1 enhancer complex to the transcription initiation complex.

5.1.3 Nature of the oxygen sensor

Evidence indicates that the hypoxia response mechanism does not react to non-specific cellular stresses but contains a specific oxygen sensor (Goldberg *et al.*, 1988). The sensing mechanism(s) appear to be specific for hypoxic stress, since induction is not seen with hydrogen peroxide, heat shock, cyanide, 2-deoxyglucose, ionising irradiation or interferon-α (Goldberg *et al.*, 1994). Hypoxia can be mimicked by carbon monoxide, by the iron chelator desferrioxamine as well as by inhibitors of heme synthesis. Induction can also be seen in response to colbalt, nickel and manganese, but not zinc, iron or cadmium. These results suggest that some component of the sensor mechanism involves a hemoprotein which changes confirmation according to its redox state; since carbon monoxide probably stimulates hypoxic induction by irreversibly substituting for oxygen and thereby preventing the porphyrin ring of the putative heme sensor from binding oxygen. The metals that mimic hypoxia are thought to displace iron in the porphyrin ring, and their low affinity for oxygen locks the heme protein in the constitutively-deoxy confirmation. Inhibitor studies also indicate that a redox-sensitive flavoprotein may operate upstream of the heme-containing sensor in this signalling cascade, since the signal generated by the formation of a putative colbalt-protoporphyrin complex is not inhibited by the flavoprotein inhibitor diphenylene iodonium (Gleadle *et al.*, 1995a). This suggests the operation of a flavoprotein oxido-reductase that is redox-coupled to the heme-containing sensor, analogous to the relationship between cytochrome P450 reductase and the heme-containing cytochrome P450s (Ratcliffe *et al.*, 1997), and raises the possibility of a CYP450 reduction product, perhaps a steroid molecule, provides a regulatory ligand for HIF-1. This would be analogous to the ligand-mediated activation of AHR by polyaromatic hydrocarbons. However recent evidence would argue against the involvement of a CYP450 monoxygenase-type oxygen sensor, in that HIF-1α itself has been identified as a non-heme iron protein. It is postulated that destabilising redox reactions may occur directly in the iron centre of the HIF-1α subunit under oxic conditions, and this Fenton-like chemistry may modify (oxidise) critical amino acids in the vicinity, targeting the transcription factor for proteosomal degradation. (Srinivas *et al.*, 1998).

5.1.4 Analysis of a candidate hypoxia responsive element.

Studies have focused on the minimal 18 bp sequence HRE isolated from the upstream promoter region (-309 to -290) of the phosphoglycerate 1 (PGK-1) gene (Firth *et al.* 1994). It has close sequence homology to the 3' HRE of Epo, with 8 of 9 b.p. being identical. This 9-bp region in the core consensus overlaps with 7 of the 8 bp in the Epo enhancer sequence shown to bind HIF-1. Gel supershift assays have demonstrated that oligonucleotides of PGK-1 and Epo HRE's cross-compete for hypoxia-induced nuclear factor binding, including HIF-1.

GGGCCCTACGTGCTGCCTCGCATGGC	EPO	mouse
GGGCCCTACGTGCTGTCTCACACAGC	EPO	human
ATTTGTCACGTCCTGCACGACGCGAG	PGK 1	mouse
GCTGCAGACGTGCGTGTG	PGK 1	human
CCAGCGGACGTGCGGGAACCCACGTG	LDH-A	mouse
TCCACAGGCGTGCCGTCTGACACGCA	GLUT-1	mouse
AGTGCATACGTGGGCTTCCACAGGTC	VEGF	rat
TCGCTTCACGTGCGGGGACCAGGGAC	ALD-A	human
CTGGCGTACGTGCTGCAG	PFK-1	mouse

Figure 1: Comparative sequence homology of HIF-1 binding sites in HREs from various hypoxia-inducible genes.

In order to test the therapeutic potential of the HRE, DNA constructs which encode either cytosine deaminase (CDase) or the cell surface protein CD2, under the transcriptional control of three promoters containing a concatamer of the PGK-1 HRE, were introduced into the human fibrosarcoma cell line HT1080. Three stable clonal lineages were generated to study the potential of HRE-driven marker gene expression in response to changes in pO_2, glucose and cAMP modulation.

The bacterial enzyme cytosine deaminase (CDase) catalyses the deamination of cytosine to uracil, and can therefore convert the cytosine analogue 5-FC into the anti-metabolite 5-FU (Huber *et al.*, 1993). It has previously been tested in a therapeutic promoter (c-erbB2) context (Harris *et al.*, 1994). Mammalian cells do not express detectable levels of CDase. 5-FU is still one of the most active single agents in the

treatment of neoplastic diseases of the colon and breast, and is commonly used in combinational chemotherapy (Scheithauer and Raderer, 1995).

5.2 Methods.

Transfection and clonal selection. Preparation of plasmid constructs and clonal selection of the HT1080 transfectants were conducted by Dr. J. D. Firth at the Institute of Molecular Medicine, ICRF, Oxford (Dachs *et al.* 1997). Briefly, a trimer of the PGK-1 HRE sequence (18 or 24 bp) was placed in the context of its homologous promoter (M3 cell line), a minimal (72 bp) thymidine kinase herpes simplex virus promoter (sTK5 cell line) or 1.8 Kba of the 9-27 gene promoter (9-3C cell line). A cell line, 2C4, which had been transfected with an identical 9-27 promoter/CD2 reporter vector, except that it lacked the triplet p18 HRE insert was employed as a control. The vectors encoded either the therapeutic enzyme CDase or the marker gene CD2. Co-transfection of both vectors was carried out for the M3 and 9-3C clonal lines, while sTK5 carried only the CD2 vector. Selection of stable clones was in 2 mg/ml Geneticin. Stable clones were tested for their response to oxygen deprivation. Multimerisation of the DNA sequence of the PGK P18+ trimer insert is shown in the context of the minimal TK promoter.

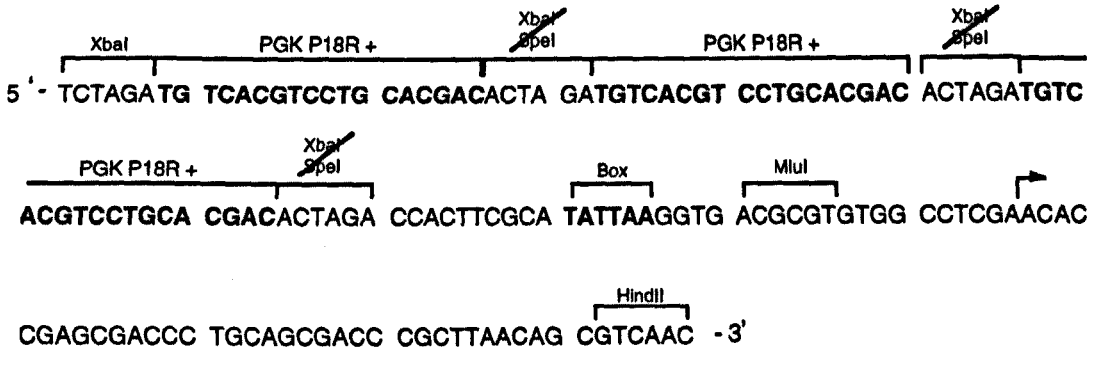


Figure 2: Short Herpes simplex thymidine kinase promoter containing three positively orientated copies of the murine PGK-1 gene hypoxia response element (p18 HRE).

Cell lines. HT1080 parental and clonal cell lines were maintained in modified minimal essential medium (DMEM). Media was supplemented as previously described.

Cytosine deaminase activity determinations. Cells of the 9-3C clonal line were exposed to 16h anoxia (palladium catalyst-induced) and reoxygenated for 5h prior to harvesting. Cells were washed in ice cold PBS, scrap ed, pelleted and resuspended in 20 µl lysis buffer (100mM Tris-HCl, 1mM EDTA, 1M DTT, pH7.8). Samples were lysed by five rapid freeze-thaw cycles, spun at 10,000g for 5 min and the S-9 fraction harvested.

10 µl samples were added to 10 µl of 100mM Tris, pH7.8, containing 5 mM cytosine-5-[^{14}C] (specific activity 0.5 µCi/10 µl). Samples were incubated at 37°C for 4h and quenched by addition of 20 µl of 100mM "cold" cytosine in 1M NaOH. 10 µl samples were loaded onto silica gel sheets and resolved in 86% 1-butanol : 14% H_2O. After drying, bands corresponding to cytosine and uracil were visualised under short-wave UV illumination and cut out. 5ml of scintillation fluid was added to each sample and total counts determined on the scintillation spectrometer. Cytosine deaminase (CDase) activity was expressed as pmol uracil produced / mg S-9 fraction / minute.

Drug sensitivity determinations. The HT1080 parental, M3 and 9-3C cell lines were tested for their response to 24h 5-FC or 5-FU exposure following a 16h anoxic or aerobic pre-treatment. After drug exposure cells were allowed to grow for a further 48h. Dose response curves were determined using the MTT proliferation assay and values of IC_{50} were determined from the individual survival curves of independent experiments. Mean IC_{50} values were derived from at least 6 independent experiments conducted on different days.

Induction of the CD2 cell surface marker. M3, sTK5 and 9-3C transfectants were tested for their response to hypoxia (0.01% O_2) and catalyst induced anoxia (<0.001% O_2) and different levels of oxygen (0.1, 1, 2, 5 and 21% O_2). The 9-3C and 2C4 cell lines were also assessed for their response to glucose deprivation, since nutrient deprivation might be expected to accompany hypoxia *in vivo*. Additionally, the modulation of PKA type I/II ratios by the therapeutic cAMP analogue, 8-Cl-cAMP was studied, in order to assess it contribution and potential application in combination with HRE-directed gene transcription. Cell surface expression of transfected reporter-CD2 was analysed using FITC-labelled anti-CD2 monoclonal antibodies (Serotec). Labelled cells were assayed for CD2 expression on a fluorescence activated cell sorter (Beckton Dickinson).

***In vivo* evaluation of drug sensitivity and CD2 expression.** The 9-3C clonal line was inoculated into the hindleg nu/nu mice. Xenografts were either treated with 5-FC (375 mg/kg, 5 x week for 3 weeks) or saline, at a starting volume of 40mm^3, or allowed to

grow to 300mm^3 (~3 weeks) and removed for histological examination with a monoclonal anti-human CD2 antibody.

5.3 Results

***In vitro* CD2 expression.** Cell surface presentation of the marker protein was monitored by labelling with FITC-labelled anti-CD2 monoclonal antibody, followed by fluorescence activated cell sorting. The results are expressed as fold-induction (O_2:N_2 ratio of median CD2 labelling) and summarised graphically in figure 3. The increase in CD2 production depended on both the length and severity of hypoxia. Following acute hypoxia (< 0.001% O_2), CD2 expression in the transfected cell lines increased during subsequent reoxygenation, whereas, following less severe hypoxic conditions, CD2 expression peaked immediately after hypoxia. This would imply a role for both chronic (diffusion limited) and acute (perfusion limited) tumour hypoxia in the induction of gene expression. CD2 expression was not induced following exposure to 5% or 20% oxygen, suggesting that physiological oxygen tensions (~ 5% pO_2) would not result in gene induction. However, some constitutive expression of CD2 was seen in the M3 and 9-3C cell lines under aerobic conditions (~ 4-fold above background auto-fluorescence). In contrast the minimal sTK promoter context (sTK5) was almost silent at 5% pO_2.

Low glucose stress (0 - 300 μM) induced CD2 expression by upto 2.2-fold in the 9-3C cell line, but not in the 2C4 control cell line lacking the HRE trimer (figure 4). Induction was less than that seen in response to 16 h anoxia, but was of similar magnitude to that observed @ 1% O_2. The combination of low glucose and hypoxia could not be evaluated, since this treatment was highly cytotoxic, and resulted in extensive cell death in both cell lines (< 5% survival after 16 h anoxia with 100 μM glucose).

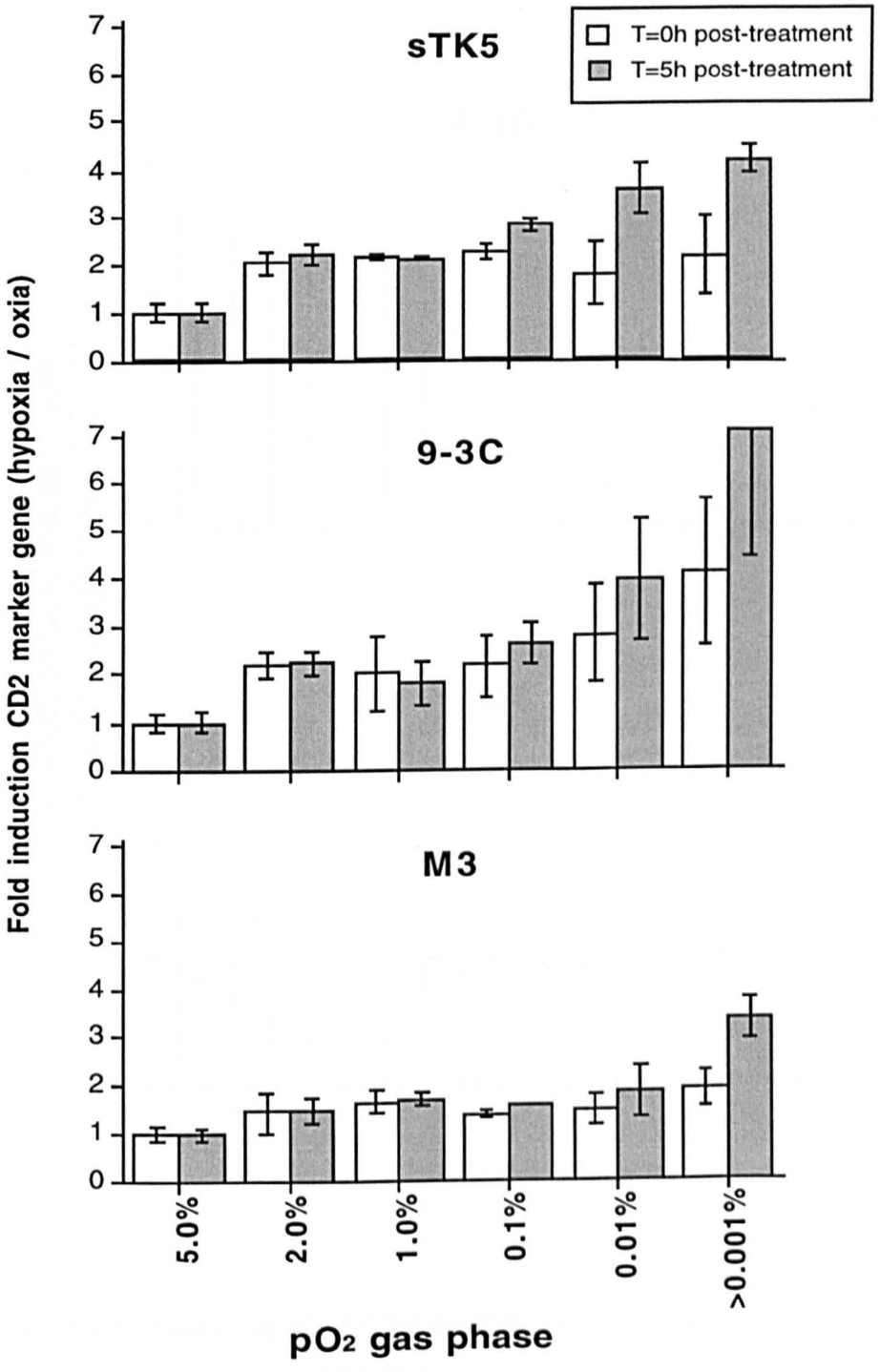


Figure 3: Increased production of CD2 by the three transfected cell lines under various gas-phase oxygen tensions. Incubations were for 16 h (clear bars) plus 5 h reoxygenation (shaded bars). The y-axis represents the ratio of hypoxic to normoxic CD2 production as determined by FACS analysis. No CD2 induction was seen in the 2C4 cell line under any conditions (data not shown).

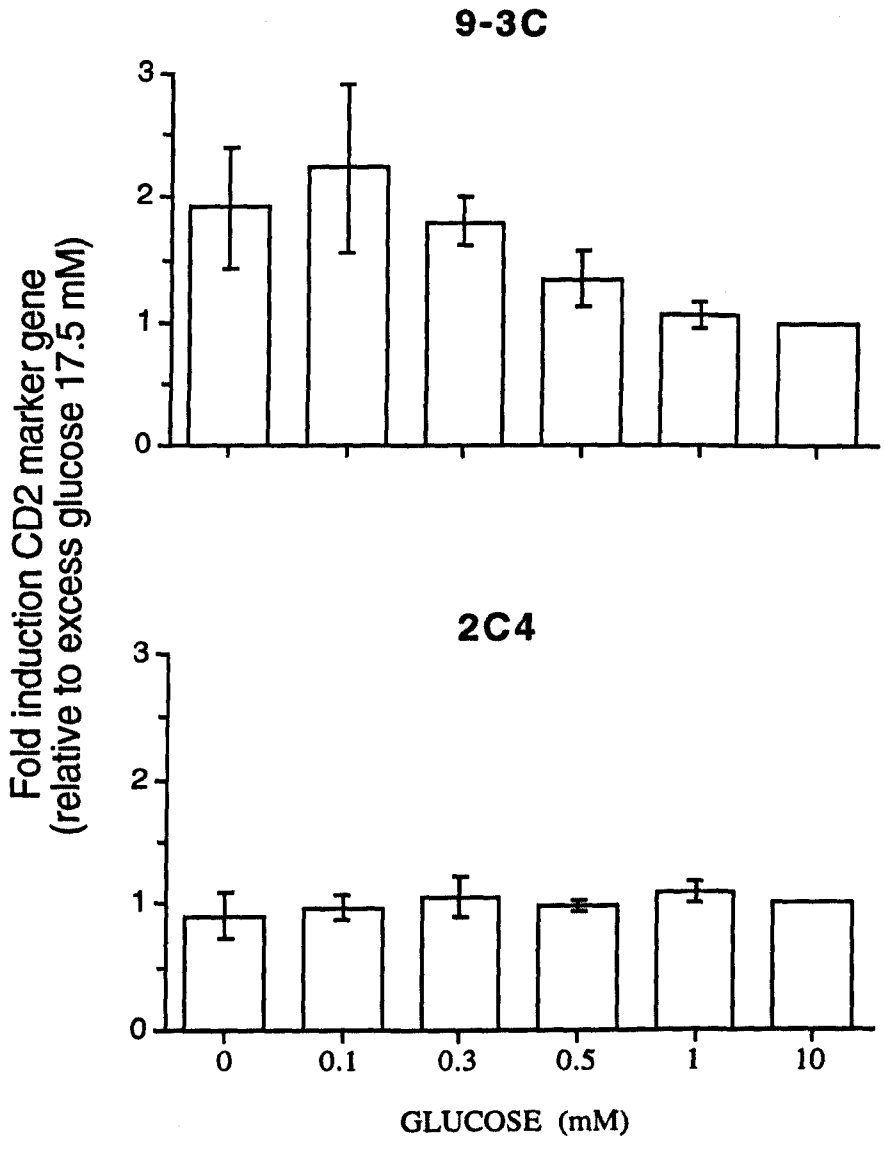


Figure 4: Increased production of CD2 by the 9-3C and 2C4 transfected cell lines under various exogenous glucose concentrations. Incubations were for 24 h. The y-axis represents the ratio of CD2 production relative to glucose-rich DMEM media (17.5 mM) as determined by FACS analysis.

The cAMP analogue, 8-Cl-cAMP, stimulated CD2 reporter gene expression in the 9-3C cell line under both normoxic and anoxic conditions (Figure 5). CD2 expression was inducted 1.9-fold under normoxic conditions and 13-fold under severe hypoxia, but this

potentiation of transcriptional activation was not seen in the control 2C4 cell line. This supports the conclusions of Kvietikova and colleagues, that there is cAMP-dependent protein kinase A involvement in the HIF-1 -mediated hypoxia signal transduction pathway.

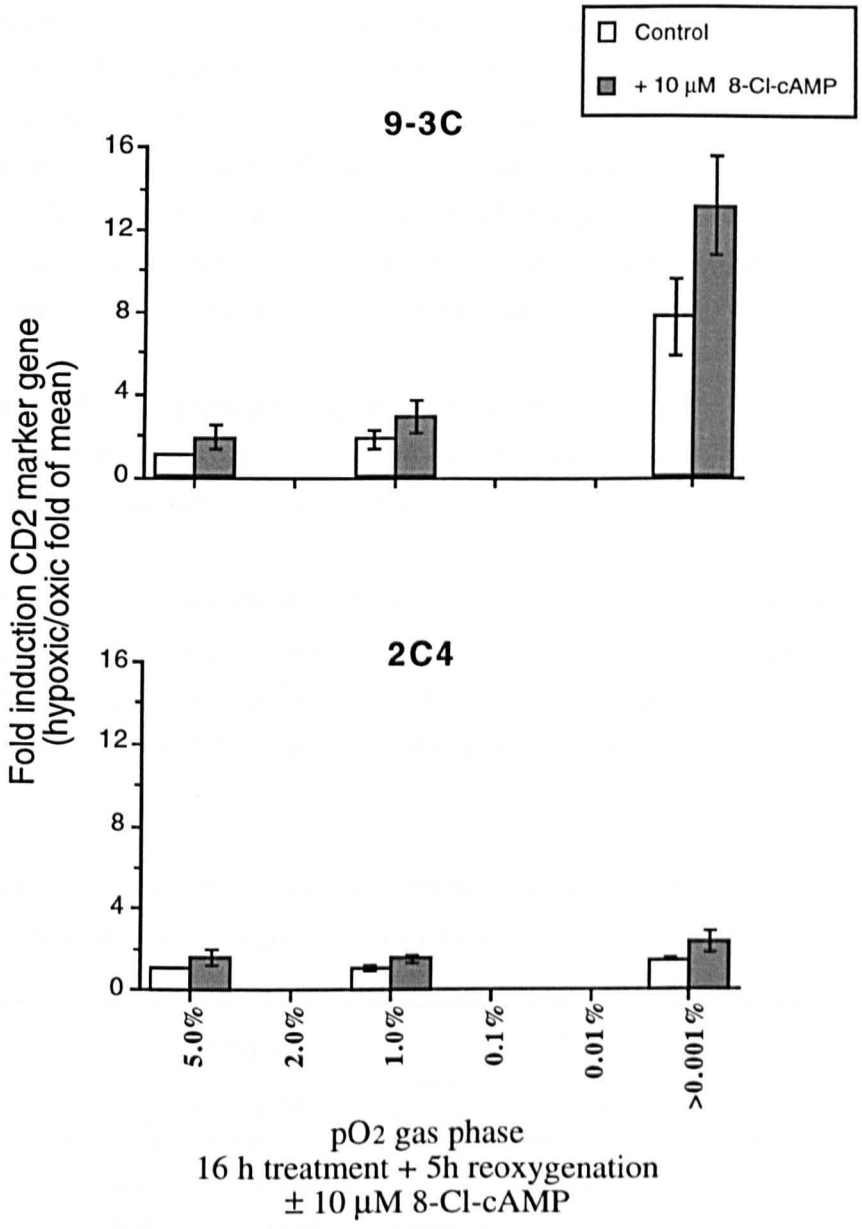


Figure 5: Increased production of CD2 by the 9-3C and 2C4 transfected cell lines under various gas-phase oxygen tensions. Incubations were for 16 h plus 5 h reoxygenation in the absence (clear bars) or presence (shaded bars) of 10 µM 8-Cl-cAMP. The y-axis represents the ratio of hypoxic to normoxic CD2 production as determined by FACS analysis.

Hypoxic induction of cytosine deaminase activity in 9-3C. Cytosine deaminase activity determinations were conducted on the parental HT1080 and the 9-3C cell lines following either oxic or hypoxic (< 1 ppm O_2) pre-treatment (16h). Initially S-9 fractions were prepared at various time points post-treatment, and the rate of uracil formation from [2^{14}C]cytosine was monitored for 1h at 37°C. Peak CDase activity was seen at approximately 4h post treatment and this time point was used in subsequent determinations. CDase activity had decreased to background levels by 28h post treatment (data not shown). No detectable CDase activity was seen in the parental cell line. The transfectant line 9-3C had detectable activity in normoxia (0.31 ± 0.04 pmol/min/mg) and showed a 6.8-fold increase in activity following 16 h hypoxia (2.10 ± 0.72 pmol/min/mg) treatment. Results are expressed as pmol uracil formed / mg S-9 protein / minute ± S.D. Results are the mean of 3 independent determinations.

The 1.8kb 9-27 promoter fragment containing a trimer of the PGK-1 p18+ HRE oligonucleutide exhibited some constitutive activity, and further transcriptional induction could be achieved following hypoxic stress.

5-FC and 5-FU drug sensitivity *in vitro*. Pre-incubation in hypoxia sensitised the 9-3C cell line 5.4 -fold (IC_{50} ratio) to 24h 5-FC exposure (figure 6). Significant sensitivity was not seen in the parental cell line. 5-FU toxicity was not significantly different for either of the cell lines following hypoxic or aerobic pre-treatment. IC_{50} data is summarised in table 1.

Table 1: Relative sensitivity of HT1080 parental and 9-3C cell lines to 5-FU & 5-FC following 16 h of aerobic or hypoxic pre-treatment.

	5-Fluorouracil IC_{50} ± s.d. (μM)		Oxic / hypoxic	5-Fluorocytosine IC_{50} ± s.d. (mM)		Oxic / hypoxic
	Post-Aerobic	Post-Hypoxic	Fold sensitivity	Post-Aerobic	Post-Hypoxic	Fold sensitivity
HT1080 wt	4.3 ± 0.31	2.9 ± 0.37	1.5	32 ± 4.3	23 ± 4.2	1.4
9-3C	1.9 ± 0.24	2.1 ± 0.29	0.9	34 ± 3.9	6.3 ± 1.4	5.4 **

** $P < 0.01$

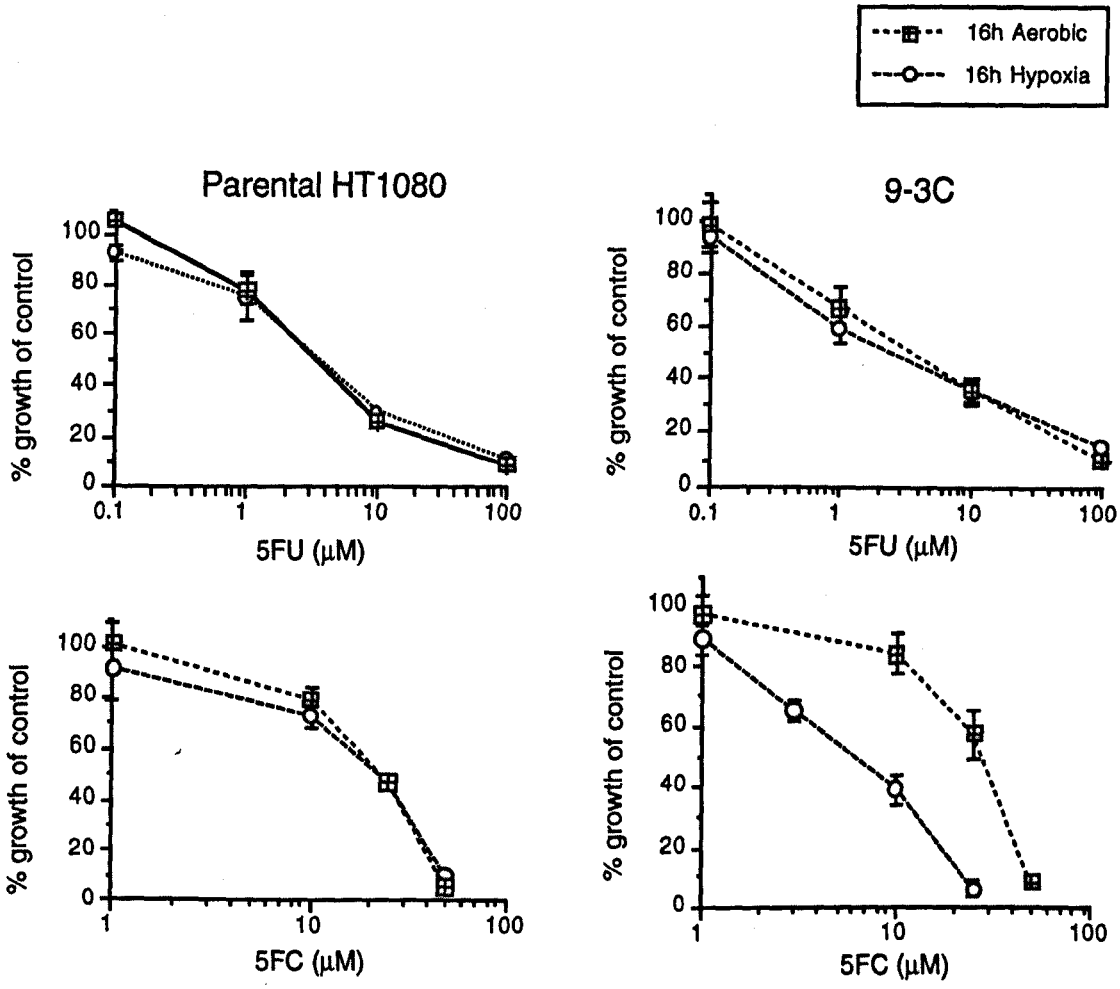


Figure 6: Representative dose-response curve of HT1080 and 9-3C cells to 5-FU and 5-FC. Exposure of 9-3C transfectants, which contain the cytosine deaminase-encoding gene under the control of the PGK-1 HRE, to anoxia before exposure to air induced sensitivity to 5-FC. Drug exposures in air for 24 h were preceded by 16 h growth in either normoxia or severe hypoxia (see legend), and sensitivity was assessed using the MTT test. Individual points are the mean of quadruplicate wells ± s.d..

***In vivo* 5-FC sensitivity and CD2 expression.** Both parental and 9-3C xenografts responded to 5-FU treatment but were not significantly sensitive to 5-FC treatment (figure 7). However, immunohistochemical analysis of serial xenograft sections revealed focal CD2 expression*in vivo* , which localised in tissues adjacent to areas of necrosis (figure 8).

An adapted alkaline comet assay analysis (Olive *et al.*, 1990) of *in vivo* xenograft cells demonstrated biologically relevant hypoxia in 10% of the tumour tissue. *In vivo* irradiation of the xenograft preferentially generated single-strand DNA breaks in cells that were normoxic at the time of treatment, while the bifunctional hypoxic cytotoxin RSU 1069 produced DNA cross-links when metabolised under hypoxia. When individual cells are embedded in agarose, lysed and subjected to an electrical current, the mobility of DNA will be enhanced by single-strand breaks but retarded by cross-linking. This allows the accurate discrimination between individual hypoxic and non-hypoxic cells from a disagregated tumour mass. When CD2 immunostaining was combined with the analysis of cellular hypoxia using the comet assay, two clearly distinct cell population were identified; short comet tails with big comet heads that were CD2-positive, and long comet tails with small heads that did not stain with anti-CD2 antibody. Thus single-cell electrophoresis of cells isolated from xenografts could be separated into hypoxic and non-hypoxic populations by means of the adapted comet assay and the sub-population identified as hypoxic were also found to be positive for CD2. This clearly demonstrated that hypoxia-regulated gene expression could be induced in individual cells *in vivo* that were radiobiologically hypoxic at the time of treatment.

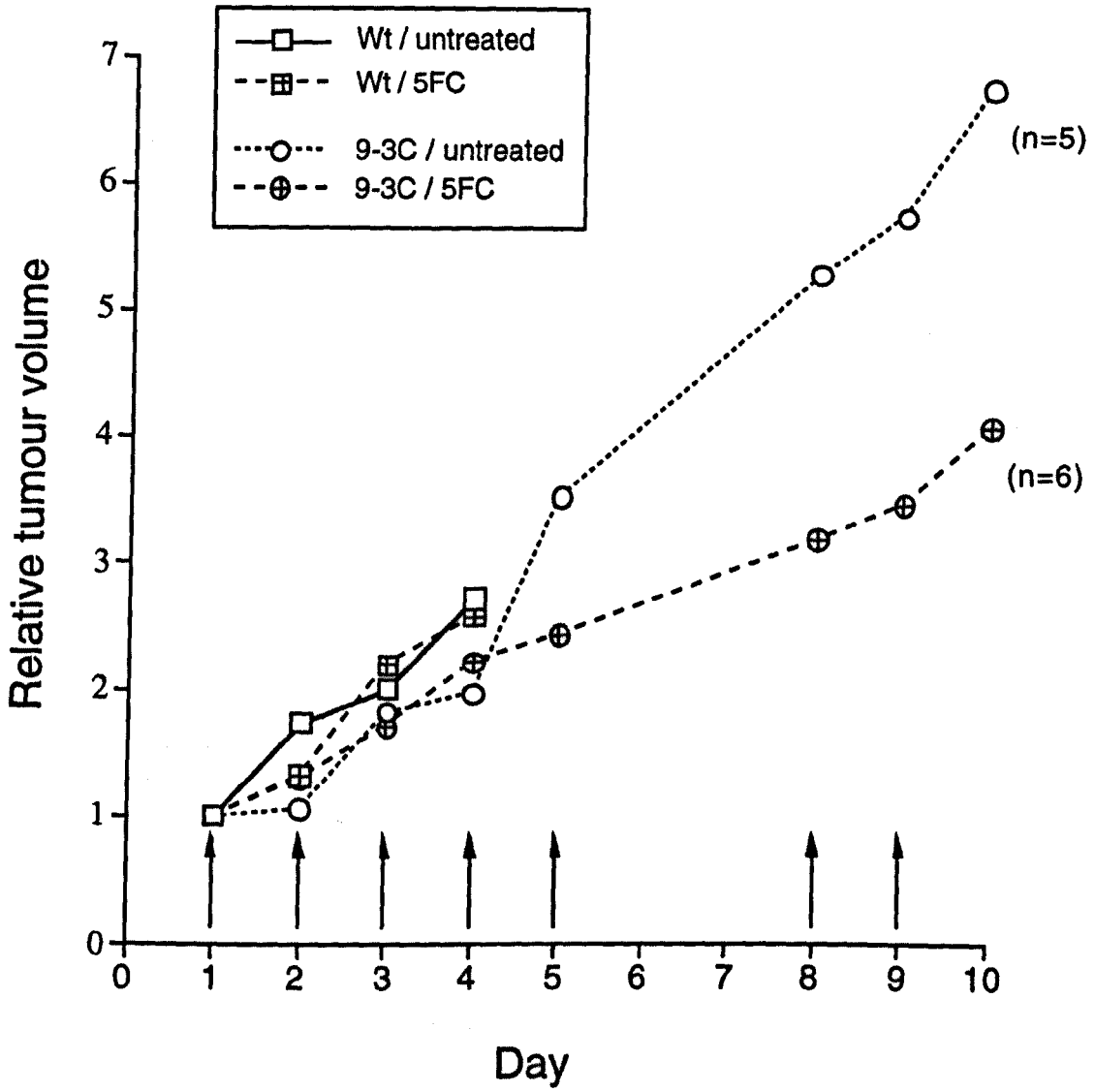
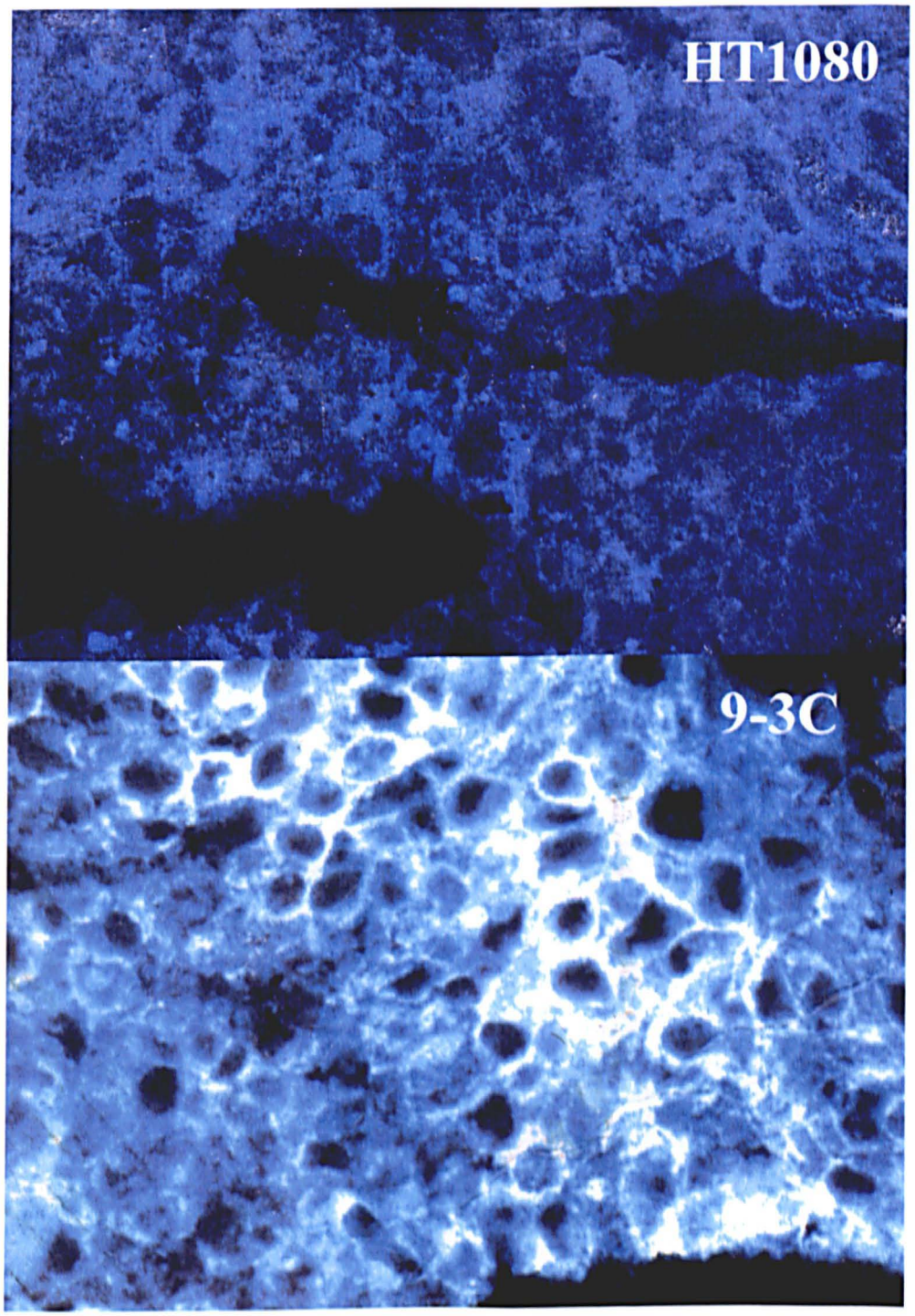


Figure 7: The parental HT1080 and 9-3C clonal line was inoculated into the hindleg nu/nu mice and allowed to establish to a starting volume of 40mm^3. Xenografts were either treated with 5-FC (375 mg/kg, 5 x week) or saline for a total of 7 days. Tumour growth was very rapid so only limited dosing could be achieved. While 5-FC treated 9-3C xenografts exhibited a modest tumour growth delay relative to saline controls, this was not statistically significant (P = 0.46).

Figure 8: Immunohistochemical analysis of CD2 expression in representative serial sections of HT1080 and 9-3C xenografts using a FITC conjugated anti-human CD2 antibody



5.4 Discussion

5.4.1 Properties of the PGK p18 HRE regulated trimer

A positively orientated trimer of the PGK-1 minimal 18 bp HRE was sufficient to confer hypoxically-regulated expression of both a reporter and therapeutic cDNA. In the CD2 reporter gene experiments the HRE trimer was found to be more active in a heterologous promoter context, perhaps suggesting that negative regulatory sequences are present in the homologous PGK-1 promoter. Constitutive aerobic expression of CD2 was very low in the minimal sTK promoter, which contrasted with the higher basal expression seen for the 1.8 kb 9-27 promoter *in vitro*. The 9-27 promoter alone was utilised in the therapeutic context of CDase expression. CDase activity could be induced 7-fold by hypoxia, and this increase in enzyme activity resulted in a 5.4-fold enhancement in acute sensitivity to the prodrug 5-FC. However, this hypoxia-inducible prodrug sensitivity was not observed *in vivo*.

CD2 induction was induced much more strongly by severe hypoxia (< 0.001% O_2) than seen at 1.0 - 0.1 % O_2, although it has been demonstrated that HIF-1 binding is maximal at ~ 0.5 % O_2 (Wang and Semenza, 1995b). This suggests that under severe hypoxia, additional signals are integrated into the hypoxic response that enhance the *trans*-activation capacity of HIF-1, independent of its DNA-binding affinity. This most likely involves redox and/or phosphorylation dependent changes. Of note, utilising Src-deficient mutant cells, Gleadle and Ratcliffe (1995) reported a functional hypoxic response at 1% O_2, while latter reports implicated a role for oncogenic v-Src in HIF-1 activation (Jiang *et al.*, 1997). It is possible that the tyrosine kinase activity of Src is only relevent to HIF-1 trans-activation at < 0.01 % O_2, since Src is also thought to participate in NF-κβ activation which only occurs at similarly low pO_2.

5.4.2 HREs and the use of cytosine deaminase-directed prodrug activation

Although the CDase / 5-FC model appeared relatively ineffective in this therapeutic context, published experiments have shown it to be an adequate enzyme / prodrug combination in other expression systems *in vivo* (Mullen *et al.*, 1994). However Mullen and colleagues reported that tumour cells escaped killing if either the levels of CDase gene expression were low or the dose or duration of 5-FC treatment was restricted. Furthermore, *in vitro* clonogenic survival experiments with 5-FC *"seemed to require*

continuous exposure to the drug for at least 5 days". This was despite the fact that the cells were sensitive to 5-FU (IC_{50} ~1μM) and the clonal line studied expressed CDase cDNA under the control of the powerful Moloney murine leukemia 5' LTR. The sharp contrast in CDase activities achievable in the hypoxia-inducible constructs relative to that of the reported MLV 5'LTR expression systems (maximal activity 1.88 *vs.* 1582 pmol uracil formed /10^6 cells / min respectively), probably explains why the 9-3C cell line did not respond *in vivo*. In support of this conclusion, the authors stated that "*successful application of the system in vivo will require maximization of intracellular expression of the enzyme as well as effective prodrug delivery*". This implies that the enzyme kinetics and 5-FC pharmacodynamics of this enzme / prodrug paradigm were poor *in vivo* - even at optimised expression and delivery parameters. This supposition is consistent with studies on the uptake of radiolabelled 5-FC, where moderate and unsaturable amounts of the prodrug accumulated intracellularly, possibly by diffusion only (Haberkorn *et al.*, 1996). This suggests that 5-FC uptake is the rate limiting factor in the treatment strategy.

Since only 10% of 9-3C xenograft tissue demonstrated biological hypoxia (≤ 0.1% O_2) *in vivo*, CDase activity would have been limited and focal. Although a bystander effect would be anticipated to occur, the relatively poor amplitude of therapeutic gene expression and thus prodrug metabolism, would preclude the possib ility of observing any measurable influence in tumour growth delay experiments.

5-FU is considered an S-phase specific drug, since its anabolites are most cytotoxic when incorporated into replicating DNA (Pinedo and Peters, 1988). *In vitro* experiments demonstrated that hypoxic treatment resulted in cell-cycle arrest and the accumulation of cells in G_1 and G_2 / M phase. Therefore 5-FU is probably not an ideal product of such an enzyme-catalysed prodrug release within the hypoxic tumour enviroment. Therefore several factors would suggest that the 5-FC / CDase system is a relatively poor prodrug / enzyme model when used in this transiently-inducible context and targeted at a growth-inhibited cell sub-population. Millimolar concentrations were required *in vitro*, and an aggressive treatment schedule was ineffective *in vivo*. Other more appropriate enzyme / prodrug paradigms may prove superior in this hypoxia-inducible GDEPT approach.

5.4.3 Manipulating the hypoxic response for therapeutic gain

The cAMP-dependent protein kinase A involvement in the HIF-1 signal transduction pathway suggests a possible method to amplify the transcriptional response of

therapeutic HREs. The PKA-dependent signal transduction pathway is a cAMP-dependent process. The cAMP molecule binds to a regulatory subunit of PKA, releasing the regulatory subunit, and PKA is activated. In mammaliancells, PKA exists as two forms, type-I and type-II, which are distinguished by their different regulatory subunits R-I and R-II that interact with common catalytic subunits, C_α, C_β and C_γ. Four isoforms of the R subunits, RI_α, RI_β, RII_α, and RII_β, have been identified. RI is found throughout the cytoplasm, whereas RII localises to nuclei, nucleoli, golgi, and to the microtubular-organising centre. C_α and C_β are associated with both RI and RII, whereas C_γ is localised with chromatin. The two PKA forms have different affinities for cAMP and different turnover rates, suggesting significant functional differences exist between them (Cadd *et al.*, 1990). Upon activation of either PKA isoform, C translocates to the nucleus and appears to be essential for the induction of cAMP-regulated genes (Roesler *et al.*, 1988). PKA stimulates the transcription of multiple genes containing CREs (Review: Lee and Masson, 1993) *via* phosphorylation of serine-133 on cAMP-responsive enhancer binding protein, CREB (Gonzalez and Montminy, 1989). CREB-1 (Habener, 1990; Lee and Masson, 1993) is known to be a target of cAMP-inducible PKA-mediated phosphorylation, which is essential for its transcriptional activator functions (Yamamoto *et al.*, 1988; Mednieks *et al.*, 1989; Nichols *et al.*, 1992).

The cAMP analogue 8-Cl-cAMP, a novel therapeutic agent currently in Phase I clinical trials, is known to modulate type I/II PKA activity ratios by substituting for endogenous cAMP, leading to differentiation and growth arrest in neoplastic tissues (Review: Cho-Chung, 1990). Its application as a hypoxia-selective co-stimuli for HRE-driven gene therapy is an attractive strategy, since 8-Cl-cAMP has growth inhibitory activity and differentation effects on a wide range of human cancer cell lines (Tagliaferri *et al.*, 1988; Ally *et al.*, 1988; Tortora *et al.*, 1988). Furthermore, it has been shown to reduce the incidence of metastatic disease in animal models (Cho-Chung, 1990), and can biochemically reverse the multidrug phenotype (Rohlff and Glazer, 1995). This is thought to involve the restoration of lower type I/II ratios, and elevated type I levels are found in several MDR cell lines (Rohlff *et al.*, 1993b). Additionally, significantly higher ratios of type I/II PKA have been found in primary breast carcinomas (Eppenberger *et al.*, 1980), which is associated with poor prognosis and reduced disease-free survival (Miller *et al.*, 1993).

8-Cl-cAMP binds with high selectivity to site B of type II protein kinase, preserving PKA-II in the holoenzyme form and so preventing proteolysis, while efficiently down-regulating PKA-I by truncating RI_α. This selectively modulates the R I/II unit ratio in

favour of RII (Ally *et al.*, 1988). This is unlike other agents that non-specifically increase intracellular cAMP levels (eg. forskolin), resulting in the simultaneous activation of both type I and type II isozymes maximally. 8-Cl-cAMP exhibits a 2.3-fold greater potency than cAMP towards PKA-I while showing 30% less potency than cAMP for type II PKA (Ally *et al.*, 1988). This contrasts 8-Br-cAMP, which not only exhibits a high-affinity for site B, but also induces potent activation of PKA-II (1.7-fold of cAMP), and is a less active growth inhibitor than 8-Cl-cAMP. Two key properties of cAMP analogues appear to determine their efficacy in growth inhibition, high selectivity towards either site A or B of RII, but not towards RI, and low activation of the PKA-II holoenzyme (Cho-Chung, 1990).

Treatment of transformed cells with 8-Cl-cAMP increases total CRE-binding activity *in vitro* (Mednieks *et al.*, 1989). Using a cAMP-unresponsive mutant PC12 cell line, it has been demonstrated that type II but not type I regulatory subunit of PKA is essential for cAMP-induced gene transcription, with RII_β being more efficient than RII_α (Tortora and Cho-Chung, 1990). Additionally, microinjection of purified C subunit has also been shown to activate CRE-containing genes (Riabowol *et al.*, 1988), and co-transfection of both C subunit and CREB genes into a cAMP-unresponsive F9 cell line induces dramatic responses with a CRE-containing reporter gene (Gonzalez and Montminy, 1989). 8-Cl-cAMP treatment stimulates the transcription and nuclear translocation of RII_β and C_α (Rohlff *et al.*, 1993a), events that are essential for the cAMP-dependent and sequence specific binding of RII_β to cAMP-responsive elements (CREs) (Wu and Wang, 1989). Following short 8-Cl-cAMP treatment, HL-60 cells exhibit a 10 and 5-fold induction in the mRNA levels of RII_β and C_α respectively, suggesting an increased transcription rate of these genes (Rohiff *et al.*, 1993a). Similarly large and selective increases in the rate of biosynthesis of RII have been observed in response to 8-Br-cAMP (Schwartz and Rubin, 1985). Overexpression of RII_β results in increased PKA-II_β holoenzyme with the concommitant down-regulation of PKA-I by sequestering the C subunit away from PKA-I. In agreement, exposure of HL-60 cells to antisense RII_β oligonucleotides resulted in decreased responsiveness to 8-Cl-cAMP-induced growth inhibition and differentiation, indicating a critical role for RII_β regulatory subunit in the cAMP-induced growth regulation (Tortora *et al.*, 1990). 8-Cl-cAMP also efficiently down-regulates RI_α by truncation of the 48 kDa RI_α subunit to a 34 kDa form, possibly by provoking the action of a specific protease. Truncated RI_α can still exist as the PKA-I holoenzyme, but truncation may facilitate rebinding of 8-Cl-cAMP, encouraging subunit dissociation and perpetuating down-regulation of PKA-I without producing accumulation of the free RI_α subunit (Rohlff *et al.*, 1993a). The rate of RI dissociation has been shown

to depend upon the relative occupancy of sites A and B by cAMP (Houge *et al.*, 1990). Suppression of RI_{α} by RI_{α} anti-sense oligonucleotides brings about a compensatory increase in RII_{β} (Tortora *et al.*, 1991), indicating that the ratio of PKA-I/PKA-II is regulated by both RI_{α} and RII_{β} availability, when RII_{α} remains constant. This might indicate that RI_{α}, RII_{α}, and RII_{β} sununits compete for a limited pool of the C subunit, and evidence suggests that RII_{α} forms the most favoured complex and serves as a reservoir of the inactive holoenzyme (Rohlff *et al.*, 1993a).

Significantly, a CRE site overlaps the consensus HRE sequence, with the core CRE motif overlapping the core HIF-1 motif ACGT (Kvietikova *et al.*, 1995). ATF-1/CREB-1 homodimers and heterodimers constitutively bind to the HIF-1 recognition site, and suggest that PKA-dependent phosphorylation of CREB may enhance HRE function. CREB phosphorylation can exert different influences on its occupancy of of high and low affinity CRE sites. PKA-dependent changes in binding of CREB increases the occupancy of weak binding sites, while high affinity sites may have constitutive binding of transcriptionally active and inactive CREB. The weak CREB binding sites are characterised by asymmetry (CGTCA) whereas the second class containing symmetrical TGACGTCA sites show a much higher affinity for CREB (Nichols *et al.*, 1992). Both human and mouse PGK HRE contain a variant of the weak asymmetrical CREB site, although the core ACGT motif is conserved between HRE and CRE.

AG**ACGTGC**	human PGK-1
TC**ACGTCC**	mouse PGK-1
TG**ACGTCA**	consensus CRE

The asymmetry of this weak CRE site within the HRE might suggest constitutive occupancy is not mediated by CREB alone, unless CREB is in an activated state. Methylation interference assays suggest HIF-1 and the constitutive bound species (ATF-1 and/or CREB-1) contact the same G residues (Wang and Semenza, 1993b), implying the possible exchange of factors upon hypoxic induction, rather than co-occupancy (Kvietikova *et al.*, 1995). Consequently, the positive influence of 8-Cl-cAMP on HRE activity under hypoxia might reflect a cooperative effect between the consitiutively bound species and the affinity of HIF-1 for the HRE site, although the direct influence of PKA on the phosphorylation status of HIF-1 can not be excluded.

Specific cooperativity between independent HRE and CRE sequences has also been demonstrated. The mouse LDH A gene is regulated by a tripartite enhancer element,

composed of a HIF-1 consensus sequence, a middle section homologous to that of Epo enhancer, and a CRE, which is essential for a functional hypoxic-response (Firth *et al.*, 1995). This implies that cAMP-dependent PKA-mediated CREB transactivation is an important signal transduction pathway that is integrated into the hypoxia response in several distinct ways. However, it is unclear whether it is the influence of 8-Cl-cAMP on the transcriptional upregulation of the C subunit, and/or the modulation of RI_{α}/RII_{β} ratios that contributes to the enhanced hypoxic response. Therefore, it is also uncertain as to whether the changes in type I/II PKA ratios contributes to this effect, which if true, could imply that PKA-II is more specifically involved in modulating the hypoxic response than PKA-I.

Overall, these observations suggest a level of integration between the secondary messenger, cAMP, and the oxygen sensor *via* cooperative transcriptional activation. This is consistent with the patterns of regulation seen for the hypoxic induction of several other genes including Epo, tyrosine hydroxylase and Glut-1, which also appear to require the cooperative interaction of HIF-1 with separate and distinct transcription factors binding at adjacent sites (Blanchard *et al.*, 1992; Huang *et al.*, 1997; Norris and Millhorn, 1995; Ebert *et al.*, 1995).

Chapter 6

6. Oxygen-sensitive GDEPT:

Analysis of potential hypoxia-dependent diffusible prodrugs for activation by the flavoprotein NADPH:cytochrome P450 reductase.

6.1 Introduction: Molecular chemotherapy.

The clinical application of sytemically delivered chemotherapy largely owes its utility to a selective toxicity towards proliferating cells. Consequently most agents are not only toxic to cancerous cells, but also to normal tissues with high rates of cell turnover. In order to achieve major therapeutic improvements it is necessary to exploit properties of neoplastic cells which are *tumour specific* rather than *proliferation specific* (Anderson, 1984). Gene-directed enzyme-prodrug therapy (GDEPT) is one such approach, where the tumour-specific activation of pharmacologically inert compounds (prodrugs), by either endogenous or specifically introduced exogenous "suicide" enzymes, brings tissue-selectivity to cancer chemotherapy. The utility of targeted chemotherapy relies upon the specificity of therapeutic gene delivery and/or appropriate neoplasia-specific gene expression. Additionally, the activated chemotherapeutic agent must elicit a tumour-localised cytotoxic effect, but equally must be capable of limited diffusion in order to target non-transfected neighbouring tumour cells.

To date the literature contains six examples of prodrugs which have been described for GDEPT in combination with non-human enzymes:

- 6-methoxy purine arabinonucleoside (ara-M) -- activated by varicella zoster virus thymidine kinase (VZV-TK) (Huber *et al.*, 1991)

- purine nucleosides; acyclovir (ACV), ganciclovir (GCV) and 1(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracyl (FIAU) -- activated by herpes simplex thymidine kinase (HSV-TK) (Moolten, 1986)

- 6-methylpurine-2'-deoxyribonucleotide (meP-dr) -- activated by *Escherichia coli* purine nucleotide phosphorylase (PNP) (Sorscher *et al.*,1994)

- 5-fluorocytosine (5-FC) -- activated by *Escherichia coli* cytosine deaminase (CD) (Mullen *et al.*, 1992; Huber *et al.*, 1993)

- dinitrobenzamide mustard derivatives (CB 1954, SN 23862 & analogues) -- activated by *Escherichia coli* B nitroreductase (NR) (Bridgewater *et al.*, 1995; Bailey *et al.*, 1996; Friedlos *et al.*, 1997)

- 5'-galactosylates of 1-β-D-arabinofuranosylcytosine (araC) and 1-β-D-deoxy-ribofuranosylcytosine -- activated by *Escherichia coli* β-galactosidase (lacZ) (Douglas *et al.*, 1991)

Several other enzyme/prodrug paradigms have been described for GDEPT, utilising human (or closely related mammalian) enzymes to activate prodrugs. The rationale for exploiting endogenous enzymes is based upon a knowledge of the rate limiting activation step(s) in prodrug activation together with the observations of limited or absent expression in neoplasic tissues. Additionally, the use of human proteins avoids the complications of acquired immunity to foreign drug metabolising enzymes, particularly following repeated administration or prolonged protein expression.

- cyclophosphamide (CPA) and ifosphamide (IFA) -- activated by rat or human cytochrome P450 (CYP) 2B and 3A respectively (Wei *et al.*, 1994; Chen and Waxman, 1995b; Chen *et al.*, 1996)

- dinitrobenzamide mustard derivatives (CB 1954, SN 23862 and related analogues) -- activated by human NADPH:cytochrome P450 reductase (P450R) (Walton *et al.*, 1989)

- 1-β-D-arabinofuranosylcytosine (araC) -- activated by human deoxycytidine kinase (dCK) (Manome *et al.*, 1996)

- 5'-deoxy-5-fluorouridine (5dFUR) -- activated by human thymidine phosphorylase (TP) (Patterson *et al.*, 1995)

6.1.1 Idealised enzyme/prodrug characteristics.

The various catalytic mechanisms of prodrug activation have been extensively reviewed in the literature (Mullen, 1994; Moolten, 1994; Conners, 1995; Springer and Niculescu-Duvaz, 1996). Only aspects that relate specifically to therapeutic efficacy will be discussed here. An appreciation of the advantages and/or limitations of each enzyme/prodrug paradigm can be considered against an idealised set of characteristics for any GDEPT system. A number of specific properties are desirable in both enzyme and prodrug components, in order to achieve the maximum therapeutic potential:

- therapeutic gene expression should be at high levels, but only in tumour cells (i.e. tumour-specific delivery and/or expression required).

- there should be low or absent background expression of any endogenous prodrug activating enzyme(s) in normal tissues.

- activation of the prodrug substrate should be direct, since any dependence for further catalysis by other endogenous enzymes might result in incomplete conversion (i.e. resistance).

- the prodrug should be freely diffusible (i.e. neutral species, appropriate partition coefficent).

- the cytotoxicity differential of the prodrug and its activated product should be as great as possible.

- the activating enzyme should rapidly metabolise its substrate (high K_{cat}) and operate efficiently at low concentrations of prodrug (low K_m).

- released drug product should have an appropriate half-life, allowing the controlled diffusion to surrounding untransfected cells that are not expressing the enzyme ("bystander effect"), while remaining sufficiently localised to prevent leakage back into the systemic circulation.

- the drug lesions should manifest their cytotoxic effects irrespective of cell cycle phase or proliferation status, so that all malignant cells will be effectively targeted.

- The relationship between drug concentration and cytotoxicity should be constant over many logs of cell kill.

6.1.2 Antimetabolite-generating systems.

Most of the enzyme/prodrug systems listed do not fulfill these idealised criteria, not least because many release antimetabolites (Springer and Niculescu-Duvaz, 1996; Kinsella *et al.*, 1997). Consequently not only do the activated antimetabolites require further metabolism by endogenous enzymes to manifest their cytotoxicity, but active DNA replication (S phase) at the time of exposure is also an essential requirement. This facilitates the incorporation of these activated nucleoside analogues into DNA, inhibiting DNA polymerase and inducing chain termination (Reid *et al.*, 1988). The dependence upon proliferation is a major limitation since the majority of neoplastic cells within a solid tumour mass will be non-cycling at any one time, making protracted or repeated prodrug administration necessary. Further, the ultimate active agent generated by these antimetabolite strategies is a charged triphosphate, which can not freely diffuse, becoming dependent upon cell-to-cell contact (gap-junction connections) to facilitate a bystander effect (Pitts, 1994; Fick *et al.*, 1995; Mesnil *et al.*, 1996). These factors are thought to contribute towards the inability of HSV-TK/GCV-mediated prodrug therapy to produce curative results in some xenografted and spontaneous mammary tumor models (Sacco *et al.*, 1995; 1996). Some of the antimetabolite prodrugs perhaps suffer from excessively long half-lives (e.g. 5-FU has a tissue half-life of several hours) (Pinedo and Peters, 1988; Kinsella *et al.*, 1997), potentially allowing the unphosphorylated drug to diffuse back into systemic circulation.

The impact of tumour hypoxia on chemotherapeutic response has been reviewed (Moulder and Rockwell, 1987; Hill, 1990; Teicher, 1994) and it is clear that the anti-proliferative effects of oxygen and nutrient deprivation (Moulder and Rockwell, 1987) can also compromise the potency of antimetabolites against this predominantly cytostatic tumour population (Kinsella *et al.*, 1997; Philips and Clayton, 1997).

6.1.3 Alkylating agent-generating systems.

The alkylating agent prodrug systems, exemplified by P4502B1/CPA and NR/CB1954, offer some significant advantages:- the DNA lesions are cytotoxic to both cycling and non-cycling cells, activation is much less dependent on other endogenous enzymes, and the cytotoxic products can diffuse across cell membranes without the need for metabolic cooperation (Douglas *et al.*, 1991; Chen *et al.*, 1996; Friedlos *et al.*, 1997). This may be a significant therapeutic advantage since the down-regulation of gap-junction function is a common feature of the de-differentiated state seen in many tumour tissues (Pitts and Finbow, 1988; Pitts *et al.*, 1988; Holder *et al.*, 1993; Pitts, 1994).

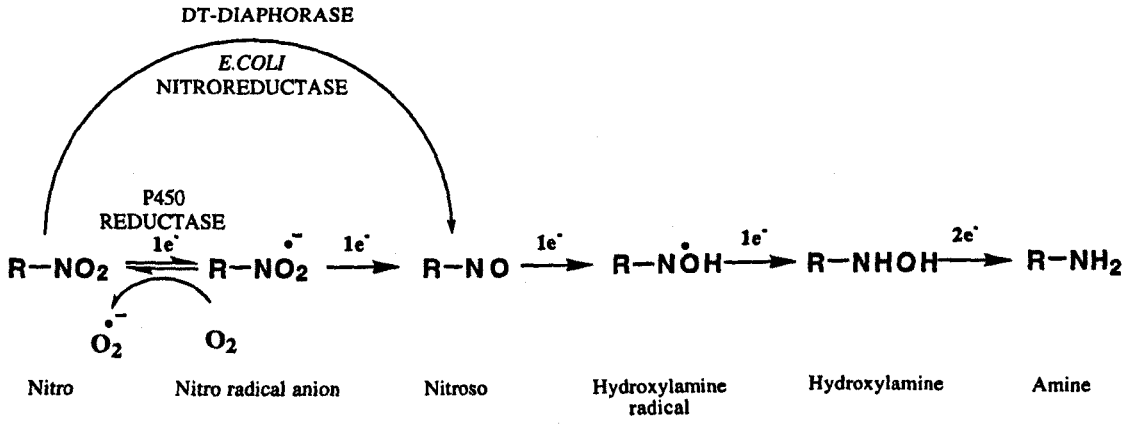
Other considerations include the *in vitro* and *in vivo* observations that alkylating agents kill cells in a log-linear manner with increasing dose of drug ($\geq$ 4 to 5 logs cell kill) whereas many antimetabolites plateau at only 1 to 2 logs (Frei *et al.*, 1988). The relative inability of cancer cells to acquire high levels of alkylating agent resistance compared to that achieved against antimetabolites (Frei *et al.*, 1988) may be clinically advantagous when applying GDEPT to heavily pretreated patients. Also, the versatility that *E.coli* NR offers by metabolising a range of nitro-prodrug substrates (Bailey *et al.*, 1996; Friedlos *et al.*, 1997) may have clinical relevance in view of the limited cross-resistance observed among alkylating agents (Frei *et al.*, 1988). Furthermore, since many solid tumours contain areas of low oxygen tension (pO_2) (chapter 1), it is relevant that alkylating agents are generally equally toxic towards normally oxygenated and hypoxic cells *in vitro* (Teicher *et al.*, 1981). CPA has even been reported to demonstrate modest selectivity for hypoxic cells (Begg *et al.*, 1985; Grau *et al.*, 1990). However, the activation of CPA by CYP450-dependent hydroxylation does itself require molecular oxygen as a cofactor (Chang *et al.*, 1993), and low tissue pO_2 will inhibit this oxidative catalysis (Jones, 1981). The improvement of tumour tissue oxygenation (by carbogen administraton) during CPA treatment appears significantly to enhance drug efficacy *in vivo* (Teicher, 1994), but this may be due, in part, to improved drug delivery to the tumour mass (Rodrigues *et al.*, 1997).

6.1.4 Utility of *E.Coli* Nitroreductase prodrugs.

Most of the proposed *E.coli* NR "nitro-trigger" prodrugs were originally developed in their own right as hypoxia-specific cytotoxins (Denny and Wilson, 1993), relying on the

natural complement of reductive enzymes in tumours to activate these agents preferentially under low tissue pO_2. The recognition that the capacity of solid tumours for reductive metabolism may be limited in some individuals (Chapter 7), has prompted the parallel development of these prodrugs as substrates for *E.coli* NR in GDEPT based therapeutic strategies (Bailey *et al.*, 1996; Friedlos *et al.*, 1997; Siim *et al.*, 1997). However *E.coli* NR-dependent prodrug activation does not exploit the hypoxic compartment of solid tumours, since it is an oxygen-insensitive, obligate two-electron reductase (Anlezark *et al.*, 1995). Consequently it reduces its nitro-prodrugs by a metabolic route that is not suppressed in oxygenated cells (see figure 1).

Figure 1: Sequential reduction of nitro-compounds by one- or two electron steps.



Thus, although these prodrugs are selectively metabolised by endogenous tumour enzymes under low tissue pO_2, *E.coli* NR does not utilise this tumour-specific property. This can have clear advantages when little or no hypoxia is present within a tumour mass, but in some cases, the application of an oxygen-inhibitable nitroreductase could provide an additional level of tumour tissue-specific prodrug activation. Providing the activated prodrug is sufficiently stable to diffuse away from its site of generation (see section 6.1.6.), the requirement for an appropriate reductive enviroment for prodrug metabolism could contribute significantly towards the therapeutic specificity of a GDEPT strategy.

6.1.5 Endogenous reductive enzymes as nitro-prodrug activators.

A variety of human enzymes can act as one-electron nitroreductases and many have quite low substrate specificities. The major oxygen-inhibitable microsomal nitroreductases are the flavoprotein NADPH:cytochrome P450 reductase (P450R) (EC. 1.6.2.4) and the hemoproteins of the cytochrome P450 superfamily (Feller *et al.*, 1971; Walton and Workman, 1987; Walton *et al.*, 1989). These membrane-associated enzymes appear to be the most important metabolic systems responsible for the reduction of model nitro-compounds, such as the 2-nitroimidazole, benznidazole (Walton and Workman, 1987). P450R dominates in the initial reduction of benznidazole to its respective hydroxylamine ($4e^-$), while cytochrome P450 (CYP450) is involved in the terminal metabolic steps to the final amine reduction product ($6e^-$) (Walton and Workman, 1987; Walton *et al.*, 1989). Benznidazole has also been implicated in the inhibition of murine cyp450 activity (Walton *et al.*, 1986). The reduction of other nitro-containing compounds has been shown to involve CYP450 (Anders and Englisch, 1985).

The molybdoflavoproteins xanthine:oxygen oxidoreductase (xanthine oxidase, XO) and aldehyde oxidase are the two major cytosolic nitroreductases found in mammalian liver (Feller *et al.*, 1971; Morita *et al.*, 1971; Wolpert *et al.*, 1973). Of these, aldehyde oxidase appears to dominate in the reduction of certain nitroimidazoles (Walton and Workman, 1987), although XO also has appreciable activity for many nitroarenes (Clarke *et al.*, 1980; 1982). However, the nitroreductase activity of aldehyde oxidase is less sensitive to oxygen than either XO or the microsomal system (Wolpert *et al.*, 1973). Xanthine:NAD^+ oxidoreductase (xanthine dehydrogenase, XDH; EC 1.2.1.37), which can be converted to XO through reversible sulfhydryl oxidation, irreversible proteolysis or tissue ischemia (Waud and Rajagopalan, 1976b; Clare *et al.*, 1981; Anderson *et al.*, 1989; Hasan *et al.*, 1991; Poss *et al.*, 1996), also has nitroreductase activity, yet like aldehyde oxidase, XDH is less sensitive to inhibition by oxygen than XO from the same source (Kutcher and McCalla, 1984). These enzymatic variations in oxygen sensitivity of nitroreduction are poorly defined, but may involve differences in oxygen competition at the substrate level, or could arise through variations in the steady-state form of the FAD prosthetic groups which can exist in equilibrium as either the flavin semiquinone ($FADH^\bullet$) or the flavin hydroquinone ($FADH_2$) species.

Nitro-compounds are generally poor substrates for the oxygen-insensitive concerted two-electron reductase DT-diaphorase (DTD) [NAD(P)H:quinone oxidoreductase; NQO1, EC 1.6.99.2] which is better known in the context of quinone reduction (Lind *et al.*, 1990; Ross

et al., 1993; Beall *et al.*, 1994). However DTD does have limited nitroreductase activity, although turnover rates are lower with nitroaromatics than with most quinones. However, significant inter-species differences exist; Rat DTD is generally a far more efficient nitroreductase than than the human form, while murine DTD has very poor nitroreductase capacity (Boland *et al.*, 1991; Beall *et al.*, 1995; Chen *et al.*, 1995c). Known substrates include CB 1954 (Knox *et al.*, 1988; Boland *et al.*, 1991; Beall *et al.*, 1994; Chen *et al.*, 1995c), 4-nitroquinoline N-oxide (De Flora *et al.*, 1988) and 1,6-dinitropyrene (Hajos and Winston, 1991). It is this limited capacity for obligate two-electron reduction of nitroaromatics by human DTD that allows the exploitation of efficient two-electron reductases such as *E.coli* NR as prodrug activators.

Of the oxygen-inhibitable nitroreductases, P450R has the most appropriate one-electron equivalent redox couples of -110, -270, -290 and -365 mV, to facilitate the transfer of single electrons to artificial electron acceptors with a range of redox potentials (Iyanagi *et al.*, 1974). Normally P450R shuttles electrons from NADPH via its FAD and FMN prosthetic groups to cytochrome P450, cytochrome b_5 or heme oxygenase (Peterson and Prough, 1986). The P450R polypeptide can be dissected into two structurally and functionally independent FAD and FMN-containing domains, each of which show striking homology to various flavin-containing bacterial proteins. The N-terminus of P450R is homologous to the FMN-containig bacterial flavodoxins, while the C-terminus is related to the FAD-containing ferredoxin $NADP^+$ reductases, strongly suggesting that P450R evolved as a fusion of two ancestral proteins. The FAD/NADPH domain is at least partly responsible for the one-electron reduction of many compounds, including redox cycling drugs (Smith *et al.*, 1994).

The redox couples of P450R correspond to the one-electron couples of the respective flavin prosthetic groups; FMN/$FMNH^•$, $FMNH^•$/$FMNH_2$, FAD/$FADH^•$ and $FADH^•$/$FADH_2$ (Vermillion and Coon, 1978a). Under aerobic conditions P450R exists exclusively as a stable semiquinone radical, probably FAD-$FMNH^•$ (Vermillion and Coon, 1978b; Yasukochi *et al.*, 1979). The air-stable reduced state of the enzyme-radical allows it to donate single-electrons to any artificial substrate with an appropriate one-electron reduction potential, the value of which will strongly determine the rates of reduction by P450R (Butler and Hoey, 1993b). This dependence on substrate reduction potential implies that thermodynamic factors predominate, and implies the interactions between P450R and its substrates are simple electron transfers which do not involve any appreciable binding of the compound to the enzyme. This capacity of P450R to facilitate the one-electron reduction of many nitro,

quinone, aromatic N-oxide and transition metal containing compounds can therefore provide oxygen-inhibitable catalysis of many reductive prodrugs (Bachur *et al.*, 1979; Powis *et al.*, 1981; Walton *et al.*, 1989; Wilson, 1992; Riley *et al.*, 1992).

6.1.6 Bioreductive drugs as hypoxia-activated diffusible prodrugs.

As an extension of the the original concept of bioreductive drugs as prodrugs activated under low tissue pO_2 to kill hypoxic cells, it has been proposed that these agents might ideally provide a bystander effect (Denny and Wilson, 1993). By releasing a diffusible cytotoxin upon reductive activation, these prodrugs could selectively utilise the small proportion of fully hypoxic cells present in most solid tumours. Of these four main redox-sensitive activation systems, only the nitro-triggered prodrugs have been extensively developed as hypoxia-activated diffusible prodrugs capable of killing oxygenated bystander cells (Wilson, 1992; Denny and Wilson, 1993; Wilson and Pruijn, 1995; Denny *et al.*, 1996).

The development of hypoxia-selective prodrugs of diffusible cytotoxins arose from the concept of exploiting the physiological (rather than genetic) differences that exist between neoplastic and normal tissue organisation, the most conspicuous of which is an inefficent vascular system (Wilson, 1992; Denny and Wilson, 1993). The hypoxic microenviroments which result from this spatially and temporally disorganised blood supply are characteristic of solid tumours of diverse origin and histology (Vaupel *et al.*, 1989), and represent an attractive approach to achieving tumour specificity through the exploitation of an oxygen-inhibitable prodrug activation. This relies on ulilising an oxygen sensitive prodrug "trigger", to allow the selective generation of diffusible products which can attack aerobic tumour cells surrounding hypoxic foci. Thus not all tumour cells need to be hypoxic if a long-range "effector" can produce a bystander effect in surrounding tissues (Wilson, 1992; Denny and Wilson, 1993). If applied in a GDEPT strategy, an additional level of specificity is introduced into the system, extending beyond that achieved by utilising promoter-specific expression or the targeting of DNA delivery. Thus in a case of aberrant (or endogenous) therapeutic enzyme expression in normal tissues, the normoxic (oxidative) tissue environment ensures that no unwanted prodrug activation occurs. Of the numerous nitro-prodrugs developed, none have yet been applied in a hypoxia-dependent GDEPT strategy.

6.1.7 Essential characteristics of the "bystander effect"

The bystander effect can be defined as an amplification of the effects of a prodrug beyond the cell in which it was activated (Pitts, 1994). Since systemic or local delivery of therapeutic genes is unlikely to target greater than 10-20% of tumour cells *in vivo*, it is considered advantagous that a prodrug kills not only cells expressing the enzyme but also surrounding non-transfected cells. Thus less than 100% infection of tumour cells can still achieve total cell kill. However, active metabolite transfer can also protect the individual drug activating cells, the so-called "Good Samaritan" effect (Wygoda *et al.*, 1997). Exploiting active metabolite transfer to induce a bystander effect might have disadvantages; it can concomitantly deliver the metabolite systemically, and there will be a large dilution effect. The half-life of activated drug is thus of considerable importance in achieving an efficient but localised bystander effect. If one considers that the diffusion range of oxygen in tumour tissue has an upper limit of ~ 200 μm, effective diffusion of a cytotoxin is unlikely to exceed this value (no matter how long-lived) since vascular clearence will interseed. Thus the diffusion range of 100-200 μm for a cytotoxin in tumour tissue would provide efficient localised cell killing with limited systemic leakage. Simplified calculations utilising Borg's equation suggest that a tissue half-live of ~ 1 minute for an activated cytotoxin would be adequate to provide an effective but localised bystander effect (assuming a diffusion coefficient in tissue of the order of 10^{-6} - 10^{-5} $cm^2 s^{-1}$) (Wilson, 1992; Denny and Wilson, 1993).

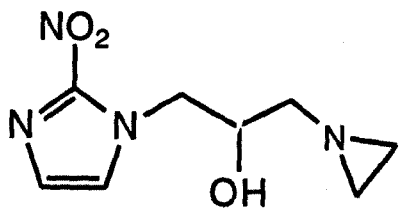
Alkylating nitrogen mustard drugs generally have half-lives in the order of minutes, but agents such as the aziridin-1-yl CB 1954 are intrinsically reactive, although a bystander effect has been observed in the majority of studies *in vitro* (Bridgewater *et al.*, 1995; Friedlos *et al.*, 1997; Green *et al.*, 1997; Friedlos *et al.*, 1998), with a notable exception *in vivo* (Clark *et al.*, 1997). Similarly, a bystander killing effect has been noted *in vitro* for the 2-nitroimidazole, RSU 1069 (Hill *et al.*, 1989). For both these agents, significant bystander cell killing *in vitro* is dependent on high cell density, suggesting the presence of a labile metabolite with relatively limited diffusion properties. This has promoted the design of reductively activated prodrugs with more stable toxic moieties (Friedlos *et al.*, 1997), or, alternatively, prodrugs that can release an oxygen-insensitive diffusible cytotoxin following enzymatic reduction (Denny and Wilson, 1993). Examples of these nitro-compounds are discussed in the next sections (6.1.8.-6.1.12.)

6.1.8 The nitro group as a latent reactive centre.

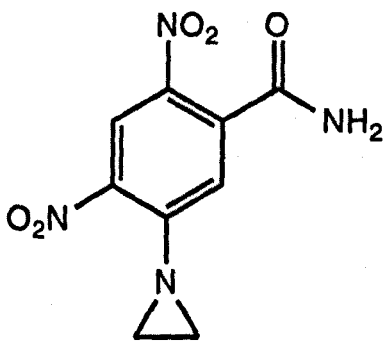
By far the largest class of bioreductive drugs are the nitro compounds, so it is pertinent to review the various mechanisms of bioreductive prodrug activation for this class of agents. Although nitro reduction products can themselves alkylate DNA, they are not sufficiently toxic to provide significant hypoxia-dependent cytotoxicity. Hypoxia-selective cytotoxicity requires that the products of net nitroreduction be more toxic than the superoxide (and resulting toxic species eg. $OH^{\bullet}$) derived from the action of the one-electron futile cycle, and also more toxic than the diversion of reducing equivalents which the latter can cause (Wardman and Clarke, 1976; Biaglow *et al.*, 1986).

If agents are of "mixed-function" containing a second monofunctional alkylating moiety such as an aziridine ring, then reduction of the nitro group serves to provide a second reactive centre. Thus under aerobic conditions aziridinyl-containing compounds will behave primarily as monofunctional alkylating agents, but the reduction of the nitro group (figure 1) will result in conversion of the molecule to a much more toxic bifunctional alkylating agent. DNA cross-links generated by such bifunctional reactivity are intrinsically more cytotoxic than the adducts arising from either spontaneous mono-alkylation through the aziridine group alone, or the reactive oxygen species generated by futile cycling (Whitmore and Gulyas, 1986; Biaglow *et al.*, 1986). Therein lies the biochemical basis for the hypoxia selective toxicity of agents such as the 2-nitroimidazole RSU1069 (Adams *et al.*, 1984) and the aziridinyl dinitrobenzamide CB1954 (Cobb *et al.*, 1969):

Figure 2: Examples of "mixed function" agents.



RSU 1069



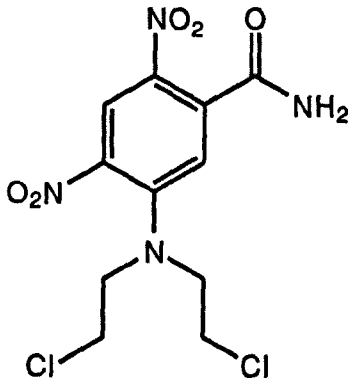
CB 1954

The actual reactive species of a sequentially reduced nitro group can vary with structural classes. With nitroimidazoles (e.g. RSU 1069) evidence suggests that the nitroso (2-e$^-$) is the key reactive species responsible for DNA alkylation (Noss *et al.*, 1989), with the nitro radical anion itself (1-e$^-$) appearing to be insufficiently reactive to be responsible for toxicity (Wardman, 1985; Boon *et al.*, 1985). For the 2,4-dinitrobenzamide, CB 1954, reduction of the 4'-nitro group to the hydroxylamine (4-e$^-$) appears to be the key metabolic step. In the case of CB 1954, the 4-hydroxylamine must also react further with cellular thioesters in order to generate the second reactive centre (Knox *et al.*, 1991). CB 1954 is now well advanced as a prodrug in combination with *E.coli* NR (Anlezark *et al.*, 1995; Bridgewater *et al.*,1995; Bailey *et al.*, 1996; Green *et al.*, 1997; Friedlos *et al.*, 1998).

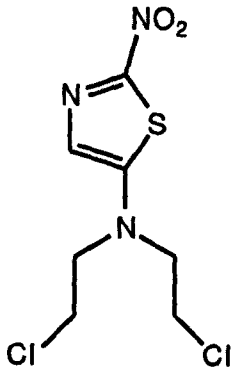
6.1.9 Nitro-reduction as an oxygen-sensitive prodrug "trigger".

Nitro group reduction is also a well-defined electronic switch that can be exploited to activate a latent alkylating function (Reviews: Denny and Wilson, 1993; Siim *et al.*, 1997). Rather than relying on enhancement of cytotoxicity through a reactive product of the nitro group itself, reduction of a nitro group is utilised to redistribute profoundly the electron density in an aromatic bioreductive molecule. The nitro group is one of the most strongly electron-withdrawing substituents in aromatic systems, while its four- and six- electron reduction products (figure 1) are strongly electron donating (Simm *et al.*, 1997). This dramatic shift in electronic ground state of aromatic molecules can be utilised to modify the reactivity of a second moiety. For example, the reactivity of nitrogen mustards are controlled almost exclusively by the electron density on the nitrogen, making it possible to prepare relatively non-toxic nitroaromatic prodrugs (Palmer *et al.*, 1990). Thus, the electron-withdrawing property of nitro substituents, when appropriately located in a ring position resonant to the mustard, ensure that the nitrogen mustard moiety is latent, but upon reduction the redistribution of electron density into the aromatic ring strongly activates the alkylating function of the mustard. This concept is exemplified by the nitrogen mustard analogue of CB 1954, SN 23862 (Palmer *et al.*, 1995) and the 2-nitrothiazole nitrogen mustard SN23727 (Siim *et al.*, 1997).

Figure 3: Examples of nitro-triggered reductively activated prodrugs.



SN 23862



SN 23727

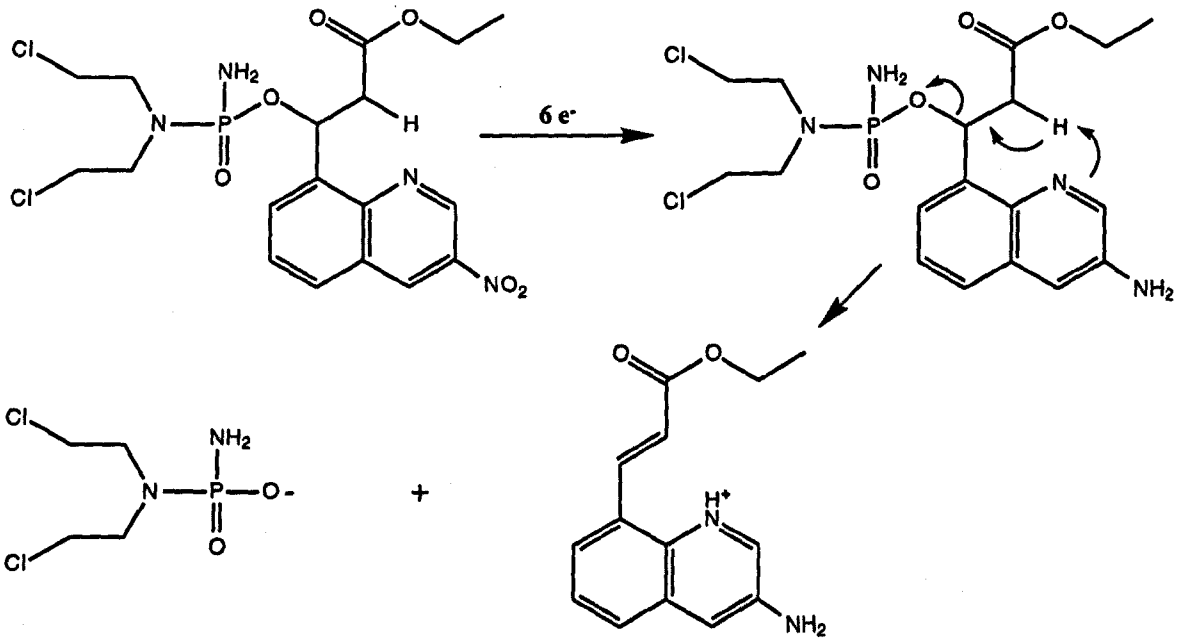
The 2,4-dinitrobenzamide nitrogen mustard, SN 23862, was designed as a less reactive analogue of CB 1954, with potentially superior diffusion capabilities following reduction (Palmer *et al.*, 1995). This supposition has proved to be valid, and subsequent bromo- and iodo- derivatives have been developed which exhibit markedly improved bystander killing capabilities over that of SN 23682 itself *in vitro* (Friedlos *et al.*, 1997).

SN 23862 requires two nitro groups on the aromatic ring in order to raise the reduction potential sufficiently to allow enzymatic nitroreduction. Generally a reduction potential in the range of -450 to -300 mV appears ideal (Denny *et al.*, 1996). Values much below -450 mV prevent significant enzymatic reduction, while agents above about -300 mV lack activity *in vivo*, probably reflecting excessively rapid rates of cellular metabolism and thus poor tissue penetration. Specific reduction of either the 2-nitro or the 4-nitro group has been shown to enhance cytotoxicity against AA8 cells by 160-fold and 9-fold respectively, while the complete chemical reduction of both nitro groups increases cytotoxicity by 1600-fold (Palmer *et al.*, 1995). However the enzymatic reduction of either one of the two nitro groups raises the reduction potential to -470 mV, limiting further cellular metabolism. Thus, enzymatic reduction of both nitro groups is not possible. Attempts to overcome this significant limitation of dinitro compounds has led to the design of mononitroheterocyclic mustards such as SN 23727 (Lee *et al.*, 1997).

6.1.10 Nitro reduction to trigger rearrangements and fragmentation.

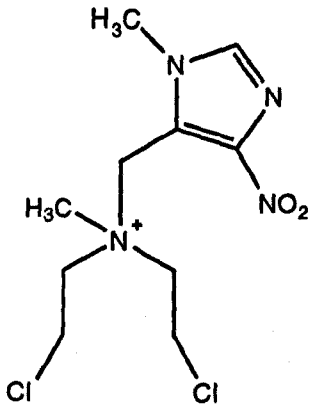
Intramolecular base-catalysed β-elimination of latent mustard groups can also be controlled by nitro reduction, as illustrated by the nitroquinilone and nitrophenyl phosporamide mustards (Firestone *et al.*, 1991; Mulcahy *et al.*, 1994).

Figure 4: Nitro reduction-dependent intramolecular base-catalysed elimination of the phosphoramide mustard from a nitroquinilone.

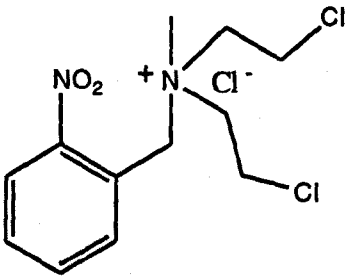


Alternatively, molecular fragmentation can be used to eliminate nitrogen mustards. For the 4-nitroimidazole-5-methylquaternary mustard SN 25341, the one-electron reduction of the nitro group to the radical anion initiates an intramolecular electron transfer event, eliminating the halide ion and forming a benzyl radical (Neta and Behar, 1980; Denny *et al.*, 1994). However, in the case of the 2-nitrobenzyl analogue SN 25246, fragmentation appears not to occur at the one-electron level (Siim *et al.*, 1997).

Figure 5: Examples of compounds employing intramolecular fragmentation to release a diffusible quaternary mustard cytotoxin.



SN 25341



SN 25246

While the mechanism of mustard release is unclear for SN 25246, it has hypoxia-selectivity up to 3000-fold in cell line culture models (Denny *et al.*, 1994; Tercel *et al.*, 1996).

6.1.11 Limitations of nitroaromatic compounds as oxygen-sensitive prodrug triggers.

Certain intrinsic drawbacks are associated with the use of nitro functionalities as "triggers" for bioreductive prodrug activation. Of clinical importance, drug formulation is hampered by low solubility, and attempts to improve the hydrophilic properties have resulted in a loss of absolute potency, providing no net gain. Crucially, nitro-groups are kinetically sluggish in electron transfer reactions, with back-oxidation by molecular oxygen being too slow to generate very large hypoxia selectivity (Wardman *et al.*, 1995). A large differential in the rate of metabolic activation (i.e. high oxygen sensitivity of reduction) is a desirable property, and it has been suggested that this is probably the limiting factor for the activity of many nitroaromatics as bioreductive triggers (Wilson, 1992; Denny and Wilson, 1993). Cumulative evidence would suggest that the hypoxic selectivities of nitroaromatic deactivated mustards appear to be limited more by the degree of oxygen-inhibition of the activation step than the absolute differential cytotoxicity between the prodrug and reduced forms. Thus, rather than increasing this differential potency, it is probably more appropriate to enhance the oxygen-sensitivity of activation. Consistent with this concept, a good correlation has been

demonstrated between the one-electron reduction potentials of various nitroheterocyclic compounds at pH 7 [E^1_7] and cytotoxic potency in Chinese hamster cell line cultures under both aerobic and hypoxic conditions (Adams *et al.*, 1980b). This suggests that nitroreduction is rate limiting for cell killing, and that mammalian nitroreductases are of sufficiently low specificity that E^1 is the primary determinant of rates of reduction and toxicity. Thus quantitatively, the dependence on E^1 was consistent with a one-electron transfer process controlling this rate. However the requirement in some cases for up to six electrons for the total reduction of a nitro group to achieve optimal activation may also limit the apparent V_{max} of prodrug metabolism. Furthermore, the terminal electron transfer events in nitro metabolism are suggested to be more dependent upon the slower reductive isozymes of cytochrome P450 rather than P450 reductase (Walton and Workman, 1987; Walton *et al.*, 1989).

Another potential limitation in the design of nitro-deactivated aromatic mustards is that the nitro 'trigger' and the alkylator 'effector' are electronically linked via the aromatic system, and yet have opposing electronic requirements. Efficient enzymatic reduction necessitates nitro reduction potentials above ca. -450 mV, which therefore requires a very electron-deficient aromatic ring. Yet aromatic mustards are relatively unreactive (i.e. when compared with aliphatic mustards), and analogues with such electron-deficient rings may have very low absolute potencies, even upon reduction of the triggering nitro group.

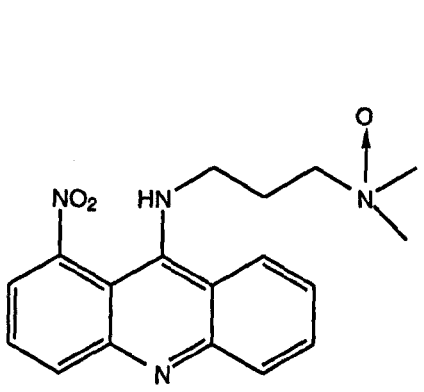
6.1.12 Other potential oxygen-sensitive bioreductive triggers.

A number of other bioreductive agents make use of oxygen-sensitive biotransformation through functional groups other than nitro, including quinones, aromatic-N-oxide, tertiary aliphatic-N-oxides and transition metal complexes (Denny *et al.*, 1996). The mechanisms of reductive activation are generally similar, although significant differences in rate constants of reduction as well as reactivity with molecular oxygen (back-oxidation) can produce quite variable properties (Wardman *et al.*, 1995).

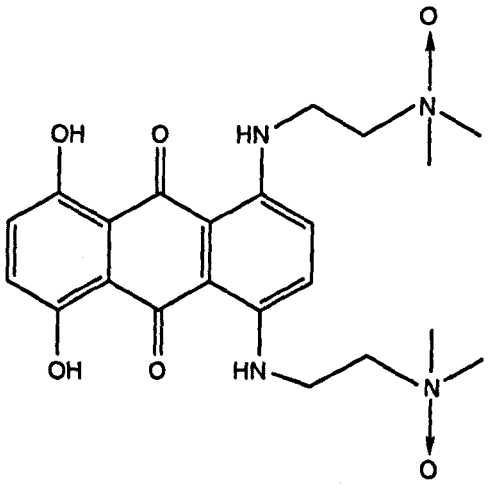
Five different redox centres have been used as reductive 'triggers' for these compounds, with the hypoxic selectivity of these agents relying upon the inactivation of a one-electron reduction intermediate through the reoxidation by molecular oxygen (Denny *et al.*, 1996). Thus the "futile cycle" that occurs in the presence of oxygen inhibits the net reduction in aerobic cells. The tertiary amine *N*-oxides, including the analogue of

mitoxantrone, AQ4N, and nitracine N-oxide are notable exceptions to this mechanism since they require concerted two-electron reduction in order to become activated (Patterson, 1993; Patterson *et al.*, 1994; Wilson *et al.*, 1996).

Figure 6: Examples of aliphatic N-oxide triggered prodrugs.



Nitracine N-oxide

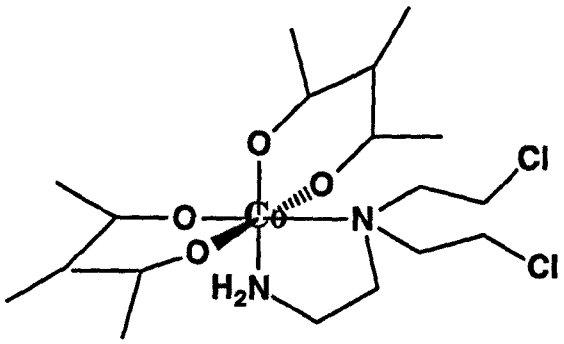


AQ4N

The N-oxides of the anthraquinones, anthrapyrazoles and anthracenes are electrically neutral and consequently are poor DNA binders and topoisomerase inhibitors. However, once reductively metabolised to their respective amines, these cationic agents become persistent, oxygen-insensitive DNA binding agents. The major disadvantage of these intercalators is their strong dependence upon cellular proliferation for cytotoxicity. It has been demonstrated that plateau-phase cells are totally refractory to this class of activated prodrug (Wilson *et al.*, 1996). In contrast, plateau-phase cells are hypersensitive to other bioreductive agents such as RSU 1069, EO9, mitomycin C and tirapazamine (Phillips and Clayton, 1997).

Despite the absence of an oxygen-sensitive one-electron intermediate, their metabolic reduction is readily inhibited by oxygen. The mechanism of oxygen inhibition of the cellular reductases is not certain, but may involve competition between oxygen and the bioreductive drug for reducing species (Patterson *et al.*, 1994).The mechanism of oxygen sensitivity for the Co(III) transition metal complexed-aliphatic mustard prodrugs is also thought to occur in a similar fashion (Ware *et al.*, 1993; Anderson *et al.*, 1996).

Figure 7: Example of a transition metal triggered prodrug.



SN 24771

To date, only the nitroaromatic and transition metal redox centres have been utilised as triggers to release deactivated effectors. Both these trigger designs have theoretical limitations, the most obvious of which is the limited oxygen-sensitivity of activation (Denny and Wilson, 1993). In principle, a quinone-triggered diffusible cytotoxin might provide certain theoretical advantages, not least the fact that quinones radicals react with oxygen around two orders of magnitude more rapidly than nitro radicals (Wardman, 1985; Wardman *et al.*, 1995). However quinones have not been studied in the context of oxygen-sensitive triggers to release diffusible cytotoxic effectors.

6.1.13 Reductive enzymes as quinone-prodrug activators.

Analagous to a nitroso radical, the distinction between a one- or two-electron reduction lies in the possibility for a single-electron reduced quinone, a semiquinone ($Q^{\bullet -}$), to redox cycle in the presence of oxygen. A concerted two-electron transfer leads to the formation of a more stable species, the hydroquinone (QH_2), bypassing the semiquinone and the possiblity of redox cycling. Hydroquinones are stable under hypoxia, and depending upon the reduction potential of the couple $E_7(Q/QH_2)$ are also relatively insensitive to autooxidation in the presence of oxygen. However a key difference between quinoids and nitro(hetro)arenes, is that the former are generally good substrates for direct two-electron reduction by the endogenous oxygen-insensitive reductase, DT-diaphorase (NQO1, DTD) (EC. 1.6.99.2) (Ernster, 1967; Ernster 1987; Lind *et al.*, 1990; Ross *et al.*, 1993, 1994; Rockwell *et al.*,

1993; Beall *et al.*, 1994; Ross *et al.*, 1996). Indeed, a large number of quinones as well as quinone imines, azo dyes and some nitrogen oxides can act as electron acceptors from DTD (Lind *et al.*, 1990; Riley and Workman, 1992b), which is a reflection of its functional relevence *in vivo*.

DTD, or more accurately NQO1, is one of four diaphorases corresponding to four gene loci (Edwards *et al.*, 1980), but interest has focused on NQO1 because it appears to be the most important for activation of various bioreductive agents and it is markedly overexpressed in many tumours and tumour cell lines (Workman, 1994; Ross *et al.*, 1994). However the enzyme is ubiquitously expressed in human tissues and often at high levels (Riley and Workman 1992b; Belinsky and Jaiswal, 1993). DTD is a predominantly cytosolic flavoprotein that exists as a homodimer, containing one FAD molecule per subunit, and is unique in its ability to utilise both NADH and NADPH as reducing cofactors (Ernster, 1967). One of its physiological roles is the reduction of vitamin K3 (menadione), which is important for biosynthesis of prothrombin and related blood clotting factors (Ernster, 1987; Lind *et al.*, 1990). Its capacity to cataylse two and four electron reductions is also important in its role as a phase II detoxifying enzyme (Ernster, 1987; Lind *et al.*, 1982). For example, the concerted two electron reduction of quinones to their respective hydroquinones allows them to be readily detoxified via conjugation with glucoronides, sulphates or glutathione, initiating elimination from the exposed cell (Lind *et al.*, 1990).

However, as implied above, the generation of hydroquinones by two-electron reduction can also bioactivate quinones to toxic species which can rearrange into potent DNA alkylating agents (Reviews: Riley and Workman, 1992b; Rockwell *et al.*, 1993; Ross *et al.*, 1993) Yet, the structure-activity relationships between DTD and the various quinone-triggered bioreductive alkylating agents has proved complex and attempts to correlate rates of reduction to the two-electron reduction potentials have proved unsuccessful (Review: Cadenas, 1995). Active site constraints appear to contribute to the substrate preferences of DTD, and the absence of the x-ray crystal structure of human DTD has, so far, precluded the ability to rationally predict and design efficient quinone substrates for DTD (Ross *et al.*, 1996). Alternatively, it is arguably more rational to evolve quinone-prodrugs that are not substrates for DTD if the intention is to specifically target tumour tissues through oxygen-sensitive bioreduction. Substrates that are bioactivated by a spectrum of one and two electron reductases, by their nature lack specificity in their application. This concept of "enzyme-directed" drug development has been suggested as a more rational way forward in the design

of novel bioreductive prodrug strategies (Workman and Walton, 1990; Workman and Stratford, 1993; Workman, 1994).

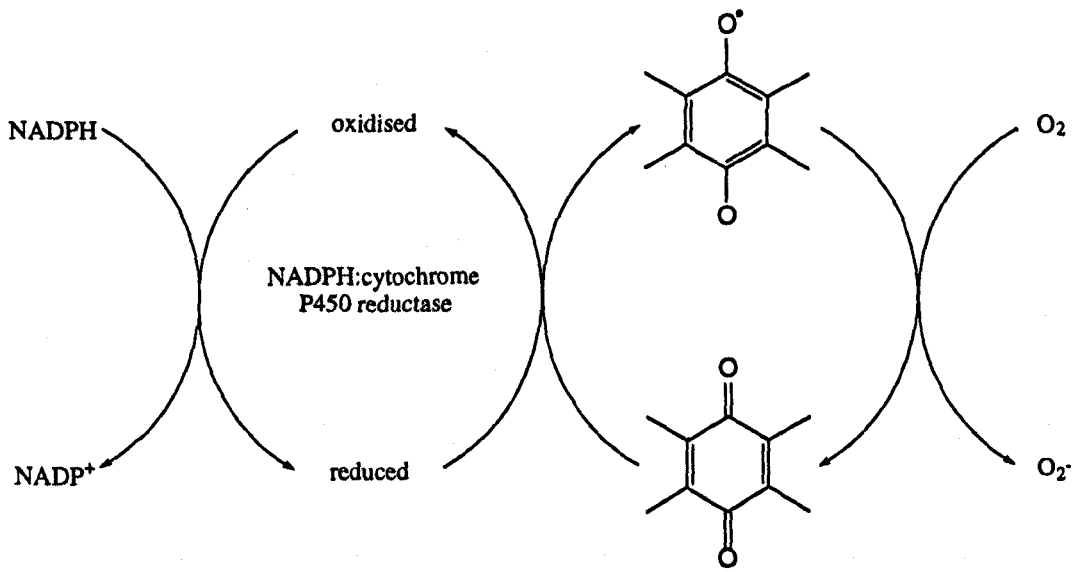
Quinones are also substrates for a variety of endogenous one-electron reductases since they will readily function as artificial single-electron acceptors (Martius, 1963). However there are no specific quinone-binding sites on these enzymes and the quinones simply divert reducing equivalents away from normal cellular redox-couples (Butler and Hoey, 1993b). The single-electron reduction potential $E_7(Q/Q^{\bullet -})$ of quinone substrates has been shown to be an important factor governing the rate of metabolism by flavoproteins that catalyse single-electron transfers (Powis and Appel, 1980; Powis *et al.*, 1981; Svingen and Powis, 1981). The semiquinone radical generated by one-electron reduction is thought to be the dominant cytotoxic species (Keyes *et al.*, 1984; Pan *et al.*, 1984; Sartorelli 1986; Plumb *et al.*, 1994b; Cummings *et al.*, 1998). In agreement, the relative cytotoxicity of a series of diaziridinyl-benzoquinones has been related to their ability to be reduced to the semiquinone form, $E_7(Q/Q^{\bullet -})$, and the intensity of the intracellular semiquinone (EPR) signals could be related to $E_7(Q/Q^{\bullet -})$ (Dzielendziak *et al.*, 1990). It was proposed that cytotoxicity resulted from the semiquinone radicals generated by the action of one-electron quinone-reductases. It has been suggested that under hypoxic conditions the free radical is sufficiently stable to enter the nucleus of a cell and interact directly with DNA (Bachur *et al.*, 1979).

The substituted 1,4-benzoquinones are the most well represented class of quinone-based chemotherapeutic agents, and their one-electron reduction is a major biotransformation that can be strongly inhibited by molecular oxygen (Reviews: Powis, 1987; Rockwell *et al.*, 1993; Ross *et al.*, 1993). The stability of a semiquinone radical in the presence of oxygen will depend upon its reduction potential $E_7(Q/Q^{\bullet -})$ relative to the oxygen couple itself, [$E_7(O_2/O_2^{\bullet -})$ = -155 mV] (Wardman, 1977; 1989), although even quinones more electropositive than -155 mV can transfer an electron to oxygen, probably because the rapid removal of the superoxide anion radical from the cell unbalances the equilibrium (Powis, 1987). Thus many semiquinone free radical forms can readily transfer their single electrons to molecular oxygen which generates superoxide and restores the original quinone (futile cycling). The superoxide radical anion is not a particularly reactive species but in the presence of catalytic amounts of transition metal ions it can give rise to secondry oxidising species, particularly the hydroxyl radical ($OH^{\bullet}$) (Halliwell and Gutteridge, 1984). The production of $OH^{\bullet}$ is more efficient under hypoxic conditions due to the direct reduction of the metal by $Q^{\bullet -}$ (Gutteridge *et al.*, 1984; Butler *et al.*, 1985). The $OH^{\bullet}$ radical is strongly oxidising, [$E_7(OH^{\bullet},H^+/H_2O)$ = + 2,180 mV]

(Koppenol and Butler, 1985), and can undergo -H abstraction or -OH addition with all biological components. However inactivation of these reactive intermediates is facilitated by various protective enzyme systems, including superoxide dismutase, catalase and the glutathione redox cycle (Review: Kappus, 1986).

Of several flavoproteins studied, NADPH:cytochrome P450 reductase (P540R) (EC. 1.6.2.4) was found to support the highest rates of single-electron quinone reduction, and this was related to the $E_7(Q/Q^{\bullet -})$ values of substrates (Powis and Appel, 1980; Powis *et al.*, 1981). The rate of single-electron reduction of simple quinones by purified P450R was found to start decreasing significantly for substrates with $E_7(Q/Q^{\bullet -})$ values below $\approx$ -250 mV, apparently tending towards background at $\approx$ -390 mV (Iyanagi *et al.*, 1974; Powis and Appel, 1980; Powis *et al.*, 1981). A range of therapeutically relevant quinones have been shown to function as single-electron acceptors for P450R-dependent electron transfer, yielding free radical intermediates (Bachur *et al.*, 1979; Svingen and Powis, 1981; Pan *et al.*, 1984; Keyes *et al.*, 1984; Butler and Hoey, 1993b). These semiquinones are poor substrates for further enzymatic reduction by P450R, but can dismutate to yield their respective quinone and the hydroquinone (Powis and Appel, 1980).

Figure 8: Schematic representation of the one-electron reduction of quinone substrates by cytochrome P450 reductase.



Putative single-electron reductases, such as microsomal NADH: ferricytochrome b_5 reductase (b_5R) (EC 1.6.2.2) (Iyanagi and Yamazaki, 1969), mitochondrial NAD(P)H: ubiquinone oxidoreductase (EC 1.6.5.3) and ferredoxin-$NADP^+$ reductase (EC 1.6.7.1) (Iyanagi and Yamazaki, 1970) can also reduce various quinone substrates, although distinctly different patterns of quinone reduction are seen (Powis and Appel, 1980). For b_5R, which only contains 1 mole of FAD per polypeptide chain, the mid-point potential for reduction is -258 mV (Iyanagi, 1977). However NAD^+ binds to reduced b_5R, stabilising the semiquinone form of the enzyme, and causes a shift in the mid-point potential to -160 mV. This binding of NAD^+ may explain the relatively high minimum potential for quinone reduction of ≈ -170 mV, with rates of reduction decreasing sharply below -100 mV (Powis *et al.*, 1981; Miller *et al.*, 1986). $NADP^+$ does not bind in this manner to reduced P450R (Iyanagi, 1977). The FMN and iron-sulphur centres of ubiquinone oxidoreductase can be reduced by either NADH or NADPH, and the pattern of substrate affinity varies with cofactor usage. NADH is the preferred cofactor and provides a mid-point potential of ≈ -230 mV. However, the maximum rate of cofactor oxidation (when coupled to quinone reduction) is ≈ 20-fold lower for NADH:ubiquinone oxidoreductase than P450R (Powis and Appel, 1980; Powis *et al.*, 1981). This is supported by studies with rotenone and antimycin A, two inhibitors of mitochondrial oxidative phosphorylation, neither of which have an appreciable effect on quinone-stimulated superoxide formation in isolated hepatocytes or mitochondrial subfractions (Powis *et al.*, 1981; Hodnick and Sartorelli, 1993). Ferredoxin-$NADP^+$ reductase can also catalyse the one-electron reduction of quinones (Iyanagi and Yamazaki, 1970), but its role in therapeutic quinoid reduction has not been studied in detail.

Several reductases have been shown to simultaneously catalyse both one- and two-electron reduction of quinones including; xanthine:oxygen oxidoreductase (xanthine oxidase, XO; EC 1.2.3.2) (Nakamura and Yamazaki, 1973), xanthine:NAD^+ oxidoreductase (xanthine dehydrogenase, XDH; EC 1.2.1.37) (Yamazaki, 1977; Gustafson and Pritsos, 1992), carbonyl reductase (Wermuth, 1981; Forrest *et al.*, 1991) and NADH:lipomide oxidoreductase (EC 1.6.4.3) (Nakamura and Yamazaki, 1972). Studies with XO comparing the specificity of one *vs.* two electron reduction have demonstrated cofactor- and pH-dependent mixed-capability for several model quinone substrates, although evidence suggests that XO preferentially catalyses the single-electron reduction of therapeutic quinone substrates (Pan *et al.*, 1984; Pritsos and Sartorelli, 1986; Butler *et al.*, 1987; Maliepaard *et al.*, 1995), In contrast, XDH clearly has both one and two electron donating capabilities with respect to therapeutic quinone substrates (Gustafson and Pritsos, 1992, 1993). Evidence for the

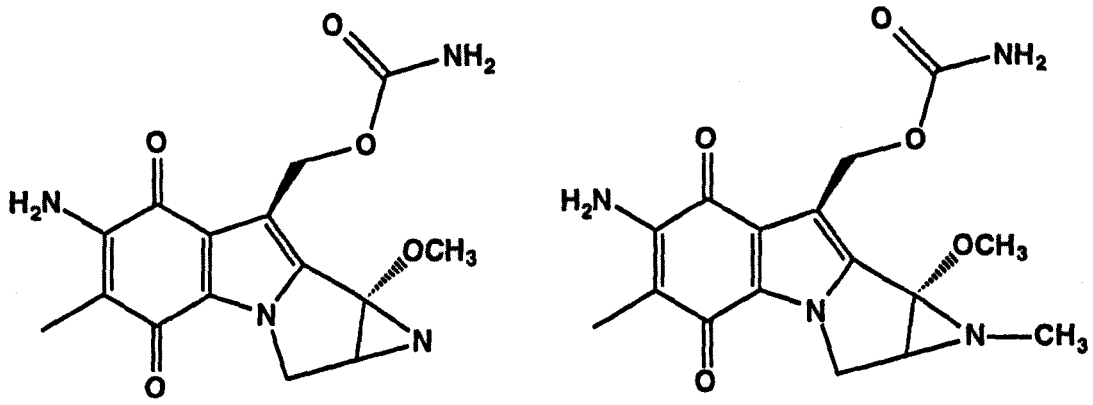
involvement of cytochrome P450 (CYP450) in the reduction of quinones is contradictary, with data supporting either a dominant role or no direct involvement of CYP450 as a quinone-reductase (Compare Keyes *et al.*, 1984; Vromans *et al.*, 1990).

6.1.14 Quinones as oxygen-sensitive alkylating prodrugs.

6.1.14.1 Mitomycins.

The quinone-containing antitumour antibiotic, mitomycin C (MMC), was in clinical use as an anticancer agent well before the development of synthetic quinones as bioreductive alkylating agents. It is the prototype for this class of agent since reduction to the semiquinone ($1e^-$) or hydroquinone ($2e^-$) is necessary to activate its alkylating propeties (Kennedy *et al.*, 1980; 1982). However the poor hypoxic differential of MMC (Rauth *et al.*, 1983; Marshall and Rauth, 1986; Stratford and Stephens, 1989) prompted the development of more selective analogues such as the N-methyl aziridine analogue, Porfiromycin (POR), which displays superior hypoxia-selectivity by virtue of a lowered aerobic cytotoxicity. This has been shown to be related to the lower incidence of DNA cross-links generated by porfiromycin under aerobic conditions (Remers, 1979; Keyes *et al.*, 1985a).

Figure 9: Examples of indole quinone-triggered antibiotic prodrugs



Mitomycin C Porfiromycin

The vast majority of therapeutic quinone metabolism in human tissues has been conducted on MMC, so it is pertinent to review our current understanding of the reductive enzymes involved, and the relative importance of one- *vs.* two-electron reductions.

DT-diaphorase. (DTD).

Considerable evidence for an association between DTD activity and MMC sensitivity has been reported in both human cell lines (Siegel *et al.*, 1990; Dulhanty and Whitmore, 1991; Marshall *et al.*, 1991; Beall *et al.*, 1994, 1995; Fitzsimmons *et al.*, 1996) and xenograft models (Malkinson *et al.*, 1992). However, MMC is preferentially activated by DTD at low pH (eg. pH 5.8) (Siegel *et al.*, 1992) owing to the pH-dependent inactivation of the enzyme by its substrate (Siegel *et al.*, 1993), and this might account for the lack of correlation between DTD activity and MMC cytotoxicity in some cell line studies (Robertson *et al.*, 1992, 1994). Recently mechanistic proof in support of the majority of these correlational studies has been provided by the overexpression of human DTD in Chinese hamster ovary (CHO) cells, which was demonstrated to confer aerobic sensitivity to MMC (Gustafson *et al.*, 1996).

However it has been noted in dicoumarol-inhibition studies that DTD may protect against MMC-induced cytotoxicity under hypoxic conditions (Keyes *et al.*, 1984, 1985b, 1989), although dicoumarol can have multiple effects on cellular metabolism, including inhibition of b_5R (Ross *et al.*, 1993; Hodnick and Sartorelli, 1993). Other evidence in support of a protective role for DTD in hypoxic cells is suggested from work on two human colon carcinoma cell lines; HT-29 with high DTD activity and BE with undetectable DTD due to a polymorphism in the NQO1 gene (Traver *et al.*, 1992). While MMC was more cytotoxic to HT-29 cells in air, only the sensitivity of BE cells was enhanced under hypoxic exposure conditions (Beall *et al.*, 1994; Plumb and Workman, 1994). Since other one-electron reductases are present at similar levels in both cell lines, it was suggested that the lack of hypoxic sensitization might imply a protective influence for DTD metabolism under low oxygen (Plumb and Workman, 1994).

P450 reductase. (P450R).

P450R is generally considered the major one-electron reductase involved in MMC activation (Kennedy *et al.*, 1985; Pan *et al.*, 1984; Keyes *et al.*, 1984; Belcourt *et al.*, 1996). Early studies with rat liver homogenates demonstrated the strong dependence of MMC metabolism on both NADPH and anaerobic conditions, with the microsomal fraction being identified as the major site of NADPH-dependent metabolism (87%) (Schwartz, 1962). A

specific role for P450R in MMC metabolism was first suggested when MMC was shown to disappear from anaerobic incubations of purified P450R and NADPH (Komiyama *et al.*, 1979). Komiyama *et al.* subsequently demonstrated that hydroxyl radicals were generated as a consequence of MMC reduction by purified P450R (Komiyama *et al.*, 1982). Later it was confirmed that there was anaerobic metabolism of MMC by purified P450R to three major products, identified using HPLC followed by mass spectrometry (Pan *et al.*, 1984). Further, the formation of an ESR signal during enzymatic reduction of MMC was seen under anaerobic conditions. This provide the first direct evidence that MMC could function as a reductively activated alkylating agent (Pan *et al.*, 1984). Soon after the importance of P450R-dependent metabolism was reaffirmed, and it was concluded that under aerobic conditions enzymatic reduction of MMC to the semiquinone radical led to redox cycling and formation of ROS, but under anaerobic conditions there was formation of an alkylating species (Keyes *et al.*, 1984). In the presence of NADPH as the electron donor, EMT6, V79 and CHO cell sonicates metabolised MMC to a product(s) capable of alkylating the model nucleophile *p*-nitrobenzylpyridine (*p*-NBP), and this reaction was strongly inhibited by oxygen. No alkylation of *p*-NBP was observed under aerobic conditions, which was consistent with observations using a reconstituted P450R system, where only 4% alkylation was observed in air (Kennedy *et al.*, 1982). These metabolism studies were consistent with the observations that P450R exists as an air-stable semiquinone radical, probably FAD-FMNH$^{\bullet}$, and thus it will exclusively catalyse the single-electron addition to an appropriate artificial electron acceptor (Vermillion and Coon, 1978b). Kinetic measurements demonstrated that no saturation of P450R activity occured, even at MMC concentrations of 5 mM (Pan *et al.*, 1984; Keyes *et al.*, 1984), consistent with the idea that there is no specific quinone-binding site on this enzyme, but rather, electron transfer is mediated by a proximity event (Butler *et al.*, 1987; Butler and Hoey, 1993b).

Surprisingly, P450R was later implicated in the activation of MMC under aerobic, but not hypoxic conditions in a CHO cell line made resistant to MMC by chronic exposure, despite a 3-4 fold lowering of P450R activity being associated with a 2-3 fold suppression of anaerobic-dependent MMC metabolism (Hoban *et al.*, 1990; Bligh *et al.*, 1990). However subsequent experiments utilising the stable expression of human P450R in CHO cells directly verified the role of this enzyme in both the aerobic and hypoxic activation of MMC and POR (Belcourt *et al.*, 1996). This pattern of both aerobic and hypoxic co-sensitisation for MMC and POR by overexpression of human P450R was later confirmed in a human mammary carcinoma line (see section 6.3.) (Patterson *et al.*, 1997).

Cytochrome P450. (CYP450).

Prior to Pan *et al.* (1984) conclusively identifying P450R as the major microsomal reductase responsible for catalysing the one-electron activation of MMC to alkylating species, it had been reported that sonicates of Sarcoma 180 and EMT6 mammary carcinoma cell lines required the presence of NADPH and anaerobic conditions to metabolise MMC, but the reduction was completely inhibited by carbon monoxide (CO) (Kennedy *et al.*, 1980). This suggested a dominant role for CYP450, and was supported by further lines of evidence in studies with reconstituted systems and isolated nuclei. It was reported that neither P450R alone, nor in combination with phospholipid, or substitution of CYP450 with cytochrome c, could result in activation of MMC (Kennedy *et al.*, 1982). However, due to a technical error, the involvement of CYP450 was later established as an indirect stimulation of P450R metabolism, with the reaction being increased upto 100% by the presence of CYP450 in a 1:1 ratio with P450R (Keyes *et al.*, 1984). Thus it appeared that CYP450 was not directly involved in the bioactivation of MMC, but under certain conditions *in vitro*, CYP450 could facilitate the P450R-dependent metabolism of MMC. This was in agreement with earlier work with a series of model quinone compounds, where no direct role for cyp450 was suggested (Powis *et al.*, 1981).

Yet, in later studies with rat liver microsome preparations, it was concluded that the dominant pathway in the one-electron reduction of MMC is via cyp450 (Vromans *et al.*, 1990). Under anaerobic conditions the initial rates of reduction of MMC by P450R was only 45% of that seen for a complete microsomal system with identical total P450R activity. Assuming first-order kinetics, the relative initial rates of reduction of MMC by P450R was 0.002 s^{-1}, but this was 100-fold lower than that of a complete microsomal system (0.20 s^{-1}). Liver microsomal H_2O_2 production was shown to be dependent on NADPH, O_2 and MMC, and could be effectively inhibited by SKF-525A and metyrapone. This inhibition was seen to be specific for cyp450, and was not thought to be caused by MMC-stimulated oxidase activity i.e. uncoupling of the cyp450 system, since reduction rates under aerobic conditions were only 10% of that under anaerobic conditions (see discussion in chapter 2). Moreover the reduction of MMC, catalysed by phenobarbitol-induced microsomal preparations under anoxia, was strongly inhibited by SKF-525A (75%) and CO (61%), although metyrapone was somewhat less effective (17%). The observation that under anaerobic conditions, the MMC-dependent alkylation of *p*-NBP was also strong inhibited by SKF-525A (70%), metyrapone (55%) and imidazole (65%), supported the conclusion that in the intact

microsomal system cyp450 was the major terminal electron recipient and donor to MMC (Vromans *et al.*, 1990). Consistent with these observations, the SKF-525A-inhibitable one-electron reductive bioactivation of 2,3,5,6-tetramethylbenzoquinone by cyp450 has also been shown (Goeptar *et al.*, 1992), and doxorubicin has been reported to be reduced to its semiquinone form by cyp2b1 (Goeptar *et al.*, 1993). However, in the absence of direct evidence for CYP450-mediated bioactivation leading to anti-tumour quinoid cytotoxicity, it is difficult to draw any clear conclusions regarding the contradictory evidence in the literature.

B_5 reductase. (b_5R)

Considering the low mid-point potential of NAD^+ bound b_5R (-170 mV), and the the observations that rates of quinone reduction decrease sharply below -100 mV (Powis *et al.*, 1981; Miller *et al.*, 1986), the relatively high one-electron reduction potential of MMC (E_7^1 = -310 mV) (Wardman, 1989), might suggest that MMC would not be a poor artificial single-electron acceptor for this flavoenzyme. In agreement, data has shown that rat liver microsomal preparations are inefficient at supporting the NADH-dependent reduction of MMC (Schwartz, 1962). However, perhaps suprisingly, purified rabbit erythrocyte b_5R has been demonstrated to catalyse the pH-dependent, NADH-supported metabolism of MMC to reactive species (Hodnick and Sartorelli, 1993). However, unlike P450R, b_5R catalysed the MMC-dependent alkylation of *p*-NPB under both aerobic and hypoxic conditions, with alkylation being ~ 1.5-fold greater under hypoxic conditions. The generation of alkylation products in air indicated that an oxygen-insensitive metabolite(s) was generated, and thus suggested b_5R catalysed a two-electron reduction of MMC, analogous to that of DTD. However b_5R also catalysed the NADH-dependent, MMC-induced consumption of O_2, indicating simultaneous one- and two-electron reductions were occuring. In further contrast to P450R, the reduction of MMC by b_5R was saturable, with an apparent K_m of 23 μM (pH 6.6).

Xanthine dehydrogenase. (XDH).

XDH is widely distributed in human tissue, and catalyses a rate-limiting step in nucleic acid degradation. MMC acts as an alternative electron acceptor, generating both oxygen radicals and the stable MMC metabolite under aerobic conditions (Gustafson and Pritsos, 1992). This is consistent with XDH being capable of bioactivating MMC by either a one- or a two- electron reduction event. Under hypoxia, rate of formation of 2,7-diaminomitosene (2,7-DM), the major metabolite of MMC, was increased 60% when xanthine was used as reducing equivalents (Gustafson and Pritsos, 1993), as compared to 120%, when NADH was the only

source of electrons (Gustafson and Pritsos, 1992). This suggested that the one-electron reduction of MMC by XDH is not as great when xanthine is used as an electron donor as opposed to NADH. Analysis of the NADH-reduced flavin in XDH has shown that the FAD existed as both the flavin semiquinone ($FADH^{\bullet}$) and the hydroquinone ($FADH_2$), with the ($FADH^{\bullet}$) predominating (Schopfer *et al.*, 1988) In contrast, xanthine-reduction favours $FADH_2$ formation. If $FADH_2$ is responsible for the two-electron reduction of MMC, and $FADH^{\bullet}$ is responsible for the one-electron reduction, the cofactor-dependence of reduced flavin products, could account for differences in the increased rates of 2,7-DM formation under hypoxia. In agreement, aerobic 2,7-DM formation was greater for xanthine- than NADH-supported MMC metabolism, implying a greater proportion of xanthine-dependent reduction is proceeding via a direct two-electron transfer (Gustafson and Pritsos, 1993). The kinetics of hypoxic and aerobic MMC metabolism by XDH appear to differ significantly. Under hypoxia, 2,7-DM formation is linear and non-saturable, similar to that seen for P450R (Keyes *et al.*, 1984). Yet, like DTD and B_5R reduction, XDH metabolism is saturable under aerobic conditions, indicated by the hyperbolic relationship between initial MMC concentration and 2,7-DM formation (apparent K_m = 299 μM; pH7.4). Of note, the rate of reduction of MMC by XDH increased as pH was decreased, and similar dependences have been noted for DTD and B_5R (Siegal *et al.*, 1990; Hodnick and Sartorelli, 1993), while the reduction of MMC by P450R is markedly decreased as pH is lowered (Kennedy *et al.*, 1985).

The combination of xanthine and NADH increases the V_{max} of XDH for a variety of substrates compared to each cofactor alone (Rajagopalan and Handler, 1967; Waud and Rajagopalan, 1976b). Thus it is suggested that the two electron donor sites are distinct, with the electron inlets for NADH and xanthine being the flavin (FAD) and molybdenum moieties respectively (Rajagopalan and Handler, 1967; Kanda *et al.*, 1972; Schopfer *et al.*, 1988). Evidence suggests that MMC is reduced at the flavin site within XDH, since xanthine does not compete with MMC at the xanthine site, but it can decrease the formation of NADH that accompanies the oxidation of xanthine to uric acid. This inhibition is competitive, suggesting MMC acts as a alternative electron acceptor to NAD^+, facilitating the removal of electrons from the enzyme (Gustafson and Pritsos, 1993).

XDH has been shown to be the precursor form of XO, which can be generated *via* the proteolytic cleavage of XDH. This XDH-to-XO interconversion is regulated through a hypoxia-dependent mechanism in tumour and normal tissues (Anderson *et al.*, 1989), Chinese hamster V79 cells (Hasan *et al.*, 1991) and hepatocytes (Wiezorek *et al.*, 1994). The

physiological role of this interconversion is poorly understood. A hypoxia-dependent rise in XO/XDH mRNA transcript levels has also been shown in endothelial cells, accompanied by increasing XO enzyme activities (Partridge *et al.*, 1992; Terada *et al.*, 1992; Hassoun *et al.*, 1994). Yet, it has also been found that ischemia-dependent increases in XO activity in endothelial cells can occur in the absence of conversion of XDH to XO, and without changes in mRNA expression (Poss *et al.*, 1996).

Xanthine oxidase. (XO).

Identification of the role of NADH-dependent reduction of MMC by the molyboflavoenzyme XO paralleled that of P450R. MMC was shown to disappear from anaerobic incubations of purified XO and xanthine (Komiyama *et al.*, 1979). Along with P450R, it was later confirmed that anaerobic metabolism of MMC by purified XO and NADH produced three major metabolites, and an oxygen-sensitive ESR signal could be generated (Pan *et al.*, 1984). As with P450R and XDH, under hypoxia the rate of MMC reduction by XO could not be saturated. The generation of ROS by XO-mediated aerobic metabolism of MMC has also been reported (Pritsos and Sartorelli, 1986), and together, these observations provided good evidence for the single-electron reduction of MMC by XO. In agreement XO-mediated reduction has been shown to be more effectively inhibited by oxygen than XDH from the same source (Kutcher and McCalla, 1984).

Earlier studies have indicated that xanthine oxidase, like XDH, catalyses simultaneous one- and two-electron reductions of several model quinone substrates (Nakamura & Yamazaki, 1973; Gustafson & Pritsos, 1992, 1993). The proportion of one-electron flux depended not only upon the source of reducing equivalents but also on the acceptor molecule itself. NADH and xanthine donate electrons at separate sites on the enzyme, the flavin and molybdenum moieties respectively, and together with the two iron-sulphur centres, this complex redox enzyme has the potential to retain upto six electrons at any one time. When NADH is the only electron donor, the % of one-electron reduction varies greatly with pH but not substrate availability, while for xanthine-supported metabolism, the % of one-electron flux is strongly dependent on acceptor substrate concentration, not pH. Since either cofactor is sufficient for activity, and the rates of xanthine- and NADH-driven reductase reactions are additive in the presence of both donors, it implies that XO has two distinct routes of intra-molecular electron transfer, although this does not necessarily imply the existence of two distinct outlets of electrons in the enzyme (Nakamura and Yamazaki, 1973). Rather, like XDH, the bifunctional reductive ability of XO more likely represents the fact that the FAD

exists in equilibrium as both the flavin semiquinone ($FADH^{\bullet}$) and the flavin hydroquinone ($FADH_2$) species. It would appear that no study has demonstrated that metabolism of MMC is truly oxygen-sensitive when xanthine is the only source of reducing equivalent.

Mitochondrial reductases.

MMC does not readily accept reducing equivalents from the mitochondrial electron transport chain (Powis *et al.*, 1981; Hodnick and Sartorelli, 1993). Recent studies have identified a NADPH-dependent mitochondrial one-electron reductase that contributes significantly to metabolism of MMC, along with the minor involvement of a NADH-dependent oxygen-inhibitable mitochondrial reductase (Spanswick *et al.*, 1996). These mitochondrial reductases appear to be more efficient than DTD at metabolising MMC (Cummings *et al.*, 1995). Importantly mitochondria have been identified as a critical target for MMC toxicity, suggesting that the activity of these reductases may be of considerable therapeutic relevance (Pritsos *et al.*, 1997).

Nuclear reductases.

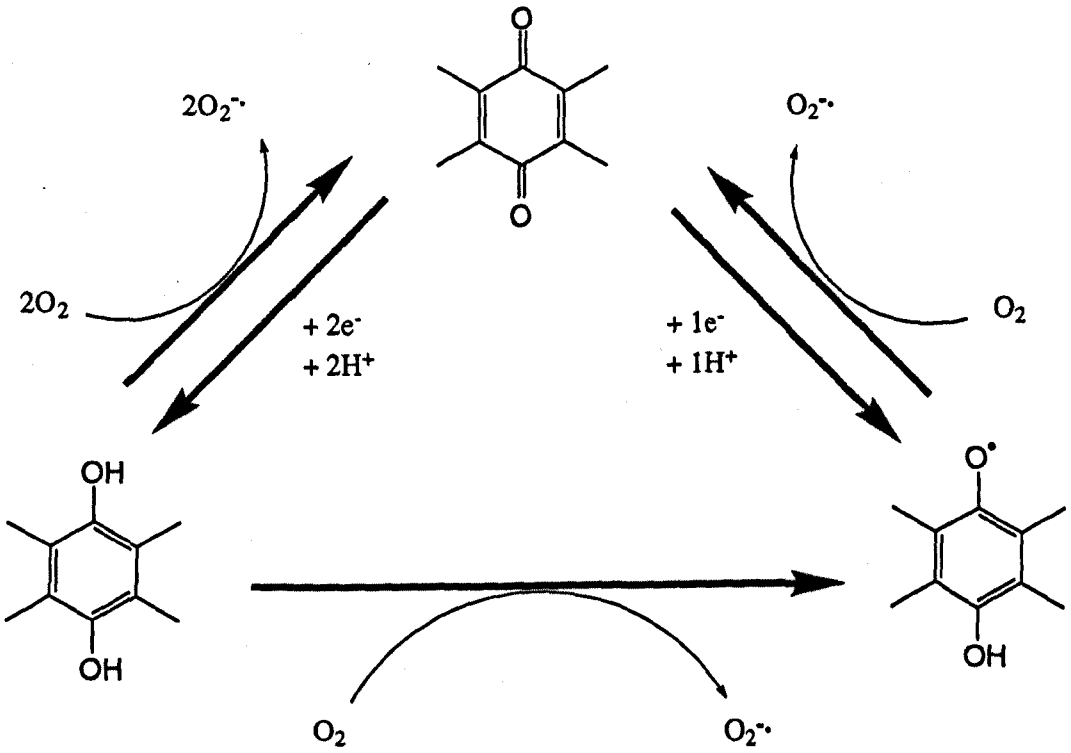
Isolated nuclei have been identified as important sites for MMC metabolism (Kennedy *et al.*, 1982; Bachur *et al.*, 1982; Keyes *et al.*, 1984; Kennedy *et al.*, 1985). Despite earlier contradictions in the literature (Kennedy *et al.*, 1982; Pan *et al.*, 1984), it was apparently clarified that P450R was the dominant nuclear reductase that metabolises MMC (Keyes *et al.*, 1984; Kennedy *et al.*, 1985). Yet the earlier suspected involvement of cyp450 in nuclear reduction of MMC (Kennedy *et al.*, 1982), coupled with later reports of the dominant role of phenobarbitol-inducible cyp450s in MMC anaerobic bioactivation (Vromans *et al.*, 1990) must leave the potential role of nuclear-localised cyp450 (Moody *et al.*, 1988), at best, uncertain.

6.1.14.2 Comparative role of one vs. two electron reduction in the initiation of MMC cytotoxicity.

It is not clear whether the one-electron or two-electron reduced forms of quinones, or both, are responsible for cytotoxicity, and mechanisms have been proposed for either the semiquinone or hydroquinone as the toxic species. The mechanism is further complicated by the potential involvement of both species with O_2-driven autooxidation, and by the possibility

of both disproportionation and comproportionation reactions between the quinones and their reduced forms (Review: Powis, 1987).

It has been suggested that the biologically active form of MMC is the two-electron reduced hydroquinone intermediate, where spontaneous elimination of methanol followed by aziridine ring opening generates a quinone methide. Nucleophilic attack by the methide initiates displacement of carbamate, providing bifunctional alkylating activity to give mitosene hydroquinone adducts (Moore and Czerniak, 1981). Yet the MMC hydroquinone has a very short half-life (≈ 0.07 sec) and rapidly and irreversibly breaksdown to a further reducible species (Rao *et al.*, 1977; Remers, 1979).



It is also proposed that single-electron reduction will serve to activate MMC. In this mechanism, semiquinone formation allows spontaneous loss of the methoxy group, forming a semiquinone radical that undergoes nucleophilic attack to give a monoadduct (Pan *et al.*, 1984; Sartorelli, 1986). Subsequent reoxidation or dismutation back to the quinone then allows

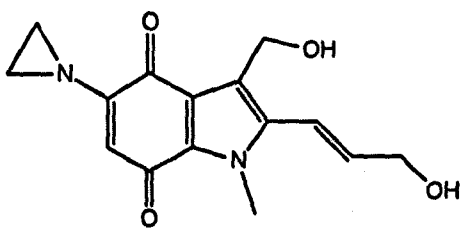
secondary radical activation to generate a bifunctional adduct (Pan *et al.*, 1986). Under anaerobic conditions the chemically generated semiquinone radical of MMC has an average lifetime of 10 seconds (Nagata and Matsuyama, 1970), although it can undergo disproportionation to the hydroquinone, so it can not be ruled out that alkylation might proceed via the two-electron pathway.

Overall, evidence favours an enzyme-mediated one-electron activation pathway since only electrochemical reduction of MMC by a single-electron gives a product profile similar to that seen for enzymatic reduction. Two-electron electrochemical reduction generates products of the 10-decarbamoyl-mitosene type that are not seen using enzyme preparations (Andrews *et al.*, 1986). Furthermore, when MMC quinone and hydroquinone are mixed together, a MMC semiquinone radical is formed by comproportionation, and it is only this semiquinone radical that can evict the methoxy leaving group (Danishefsky and Egbertson, 1986).

6.1.14.3 Indoloquinones: EO9 and its analogues.

A synthetic analogue of MMC, the aziridinylindoloquinone, EO9 (Oostveen and Speckamp 1987), was found to have a much superior hypoxia selectivity than MMC *in vitro* and *in vivo* (Robertson *et al.* 1992, 1994; Adams 1992; Hendriks *et al.* 1993). EO9 has significant structural changes over MMC, of which the aziridinyl moiety at the 5-position of the quinoid ring is the most important in terms of cytotoxicity (Bailey *et al.*, 1992; Phillips 1996; Jaffar *et al.* 1998A).

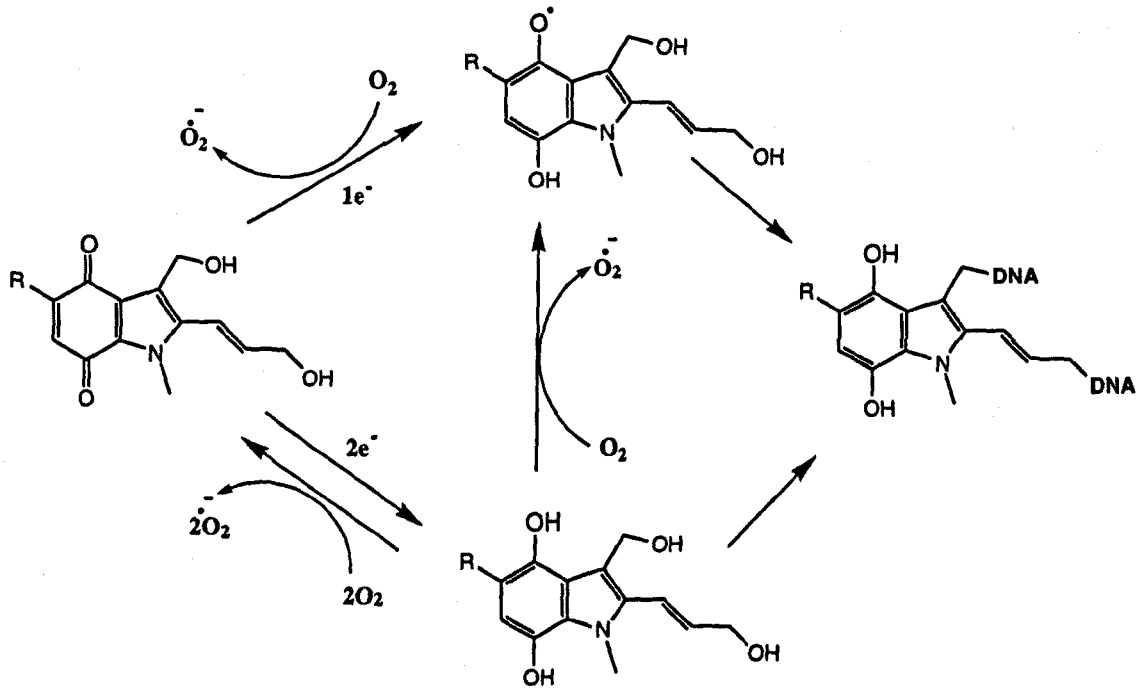
Figure 10: The indoloquinone EO9: [3-hydroxy-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)-prop-β-en-α-ol].



EO9

EO9, like MMC, is subject to both one- and two- electron reductive metabolism to the semi- and hydroquinone respectively, although details of the relative contributions of cellular reductases, and their respective contributions to aerobic vs. hypoxic cytotoxicity, have only recently begun to emerge.

Figure 11: Proposed differential oxygen-sensitivity of indoloquinone metabolism by one- or two- electron reduction pathways.



Early studies identified DTD as an imporant enzyme for the metabolism of EO9, and bioactivation of EO9 by purified DTD resulted in DNA single-strand breaks (Walton *et al.*, 1991). The suggestion of a role for DTD in EO9 cytotoxicity was confirmed by Robertson *et al.* (1992), when a good correlation was demonstrated between the sensitivity of a panel of human cell lines to aerobic EO9 exposure with intracellular DT-diaphorase activity *in vitro*. A number of studies have since reaffirmed this relationship (Walton *et al.*, 1992b, 1992c; Robertson *et al.*, 1994; Smitskamp-Wilms *et al.*, 1994; Plumb *et al.*, 1994a; Collard *et al.*, 1995; Fitzsimmons *et al.*, 1996), as well as confirming EO9 to be a relatively good substrate for human DTD (Beall *et al.*, 1994, 1995; Chen *et al.*, 1995c; Phillips *et al.*, 1996; Bailey *et al.*, 1997). Reduction of EO9 by purified DTD has been shown to result in either DNA single-strand breaks (Walton *et al.*, 1991; Phillip 1996), or DNA alkylation and cross-linking (Bailey *et al.* 1994a; Maliepaard *et al.* 1995; Bailey *et al.*, 1997) and as such there is disagreement in

the literature as to the nature of the cytotoxic lesions generated under aerobic conditions (Butler *et al.*, 1996; Cummings *et al.*, 1998). Butler *et al.* (1996) demonstrated that the hydroquinone was rapidly backoxidised to EO9 under aerobic conditions ($t_{½}$ =1.5 sec) with the concomit ant production of H_2O_2, suggesting ROS formation (particularly $OH^{\bullet}$) would be the dominant cause of DNA damage. This must question the ability of the hydroquinone, formed directly by DTD-dependent metabolism, to generate significant crosslinking in intact cells, and suggests in studies where correlations were observed between the cytotoxicity of EO9 in air and DTD expression, the effect was mediated via ROS. Yet, Walton *et al.* (1991) observed that superoxide dismutase did not prevent the formation of single strand breaks, implying that alkylation was initiating the DNA damaging effect. Whatever the mechanism, direct evidence that human DTD is relevant to EO9 toxicity in whole cells has recently been provided by transfection of human NQO1 cDNA into CHO cells. Clones expressing high levels of enzyme were modestly more sensitive (3-fold) to aerobic EO9 exposure (Gustafson *et al.*, 1996).

Robertson *et al.* (1994) also demonstrated that in cells with high DT-diaphorase, treatment with EO9 under hypoxic conditions did not provide significant additional cytotoxicity, whereas cells with low DT-diaphorase were markedly sensitized to EO9 in hypoxia. This is consistent with a dominant oxygen-insensitive role for DT-diaphorase in the metabolism of EO9 (Walton *et al.*, 1991; Bailey *et al.*, 1994b), but in the absence of significant direct two-electron reduction, alternative oxygen-sensitive one-electron processes will contribute to cytotoxicity, but only under low oxygen tensions. In agreement, it has been demonstrated that an inverse correlation exists between the HCR of EO9 and DT-diaphorase activity in two independent panels of human cell lines (Plumb *et al.*, 1994a; Robertson *et al.*, 1994). However, like MMC, increasing DTD levels were also shown to actually confer protection from EO9 cytotoxicity under hypoxia, and this correlation was lost in the presence of dicumarol (Plumb *et al.*, 1994b). Plumb *et al.* (1994a) suggested that back-oxidation of the hydroquinone to the significantly more toxic semiquinone radical is prevented by the absence of oxygen, and this possibility, together with supporting evidence that the semiquinone is the proximate toxin, implied that direct two-electron metabolism of EO9 by DTD under hypoxic conditions is essentially a detoxifying process. If these hydroquinone reactions are not directly damaging, then this process will be in competition with the major cytotoxic single-electron reduction pathway.

The importance of one-electron reductases in the bioactivation of EO9 was exemplified using the HT-29 and BE cell line pair (Plumb and Workman, 1994; Plumb *et al.*, 1994b). Under hypoxia, the DTD-rich HT-29 cells demonstrated only a 2.9-fold increase in cytotoxicity, while the DTD-deficient BE cell line became 1000 to 3000 -fold more sensitive to EO9 exposure. The relationship between aerobic potency of EO9 has subsequently been correlated to DNA damage in these cell lines (Bailey *et al.*, 1997). BE cells showed a dramatic 30-fold increase in the extent of crosslinks formed under hypoxic conditions, supporting the relevance of one-electron reduction in EO9 cytotoxicity under low oxygen tension.

Perhaps more importantly, *in vivo* studies have also demonstrated that EO9 functions as a hypoxia-selective cytotoxin (Adams *et al.*, 1992). While EO9 was inactive against the KHT sarcoma in mice as a single agent, it could strongly potentiate the action of 10 Gy X-irradiation. This effect was much larger for EO9 than MMC, and appeared to be related to the superior hypoxic cytotoxicity ratio of EO9 against KHT cells *in vitro*. It was concluded that since 10 Gy was sufficient to eradicate the aerobic fraction of the xenograft, this implied that EO9 was functioning as a hypoxic cytotoxin. In support, a greater than additive effect was seen in four types of rat tumours when EO9 was administered postirradiation (Kal *et al.*, 1994, 1995). Two *in vivo* studies have attempted to correlate tumour response to EO9 with reductive enzyme profile, but no relationship has been found for either DTD (Collard *et al.*, 1995; Cummings *et al.*, 1998), P450R or b_5R activities (Cummings *et al.*, 1998). However in the latter study, tumour sensitivity *in vivo* was shown to be related to the overall ability of tumour homogenates to catabolise EO9 (Cummings *et al.*, 1998) under hypoxic conditions (r^2 = 0.86; P = 0.07), but not so clearly under aerobic conditions (r^2 = 0.82; P = 0.17). However, since the severity and extent of hypoxia within these xenograft models was not determined, interpretation of the data is difficult.

The specific role of P450R in the hypoxia-dependent bioactivation of EO9 has been confirmed in cell free studies (Bailey *et al.* 1994a). ESR spectroscopy experiments using purified P450R detected EO9 and O_2 radical species, while incubation of P450R, NADPH and EO9 with marker DNA lead to the generation of DNA interstrand crosslinks, but only under anaerobic conditions. To date, the relevance of P450R has not been demonstrated in intact cells.

Purified XO has also been shown to activate EO9 to yield DNA interstrand crosslinks (Maliepaard *et al.* 1995). Incubations were only conducted under anaerobic conditions using

NADH as the source of reducing equivalents. Conversion of EO9 was increased with lowering pH, but less DNA cross-linking resulted. XO-activated EO9 was shown to be a more potent cross-linker than activated MMC under identical experimental conditions. However, no evidence was reported to confirm that XO does not metabolise EO9 to a cross-linking species in aerobic conditions, and neither was xanthine employed as an alternative electron donor. Thus it is not possible to conclude whether XO-mediated activation of EO9 is, like MMC, apparently an exclusive single-electron reduction event.

6.1.15 Quinones as oxygen-sensitive triggers.

While quinones and nitro groups appear to follow similar overall kinetics, being strongly dependent upon the substrate reduction potentials (Dzielendziak *et al.*, 1990; Butler and Hoey, 1993b), the energetics controlling the rate of reaction of the two types of drug radicals with oxygen are significantly different. Quinones have low mid-point potentials (E^1_m) for the one-electron couples, and the rate constants are not much lower than the diffusion-controlled limit. In contrast, for similar energetics, nitro radicals react with oxygen around two orders of magnitude more slowly than quinone radicals (Adams *et al.*, 1980b; Wardman, 1985; Wardman *et al.*, 1995). Since the reaction of the radical with oxygen will also be in competition with radical disproportionation, a pathway which leads to toxicity, the rate of change of radical concentration with time will be, in simplest terms, an opposing balance of these two factors. For similar energetics, quinone radicals disproportionate up to four orders of magnitude faster than nitro radicals, but the oxygen sensitivity of this process is dependent upon the square of this rate constant (Wardman *et al.*, 1995). However, since this difference is offset by the ≈100-fold greater rate constants for reaction of quinone radicals with oxygen compared with nitro radicals, the overall kinetic basis for the use of either nitro or quinone based agents as bioreductive drugs have evident parallels. Yet the absolute rate constants for their individual reactions differ by orders of magnitude.

6.1.16 Implications for the oxygen concentration dependence of bioreductive drug metabolism.

The marked sensitivity of quinone substrates to back-oxidation is reflected in their 'K-curves' or oxygen dependence of cell killing. Generally the half value ('K-value') for most

nitroimidazoles is ≈ 0.5 - 0.1% O_2 (Koch, 1990; Mulcahy, 1984; Brown, 1993; Koch, 1993), while for quinone bioreductive drugs such as mitomycin C or diaziquone the K-value is in the order of 0.01% O_2 or less (Marshall and Rauth, 1986; O'Brien *et al.*, 1990).

The need for severe hypoxia to achieve significant reductive metabolism of quinones when compared to nitrocompounds has obvious clinical disadvantages when considering their application as bioreductive drugs. Since the K-value for radiation sensitization by oxygen is ≈ 0.3% (Hall, 1994), the use of bioreductive drugs that require significantly more stringent hypoxia for maximal efficacy will leave cells at intermediate oxygen concentrations resistant to both the bioreductive agent and the radiation treatment (Brown, 1993). However, this acute hypoxia-dependence might also represent an advantage when considering the tissue specificity of quinone reduction, since the low oxygen concentrations required are unique to tumour tissues. Intermediate oxygen tensions are a common physiological property of several normal tissues, and have been associated with the severe side-effects of bioreductive drug therapy. For example, TPZ induces acute liver toxicity in rat models (White *et al.*, 1992), and the resulting liver necrosis is confined to hepatocytes in the pericentral zone, a region that only experiences oxygen tensions of 2 - 4% (Lemasters *et al.*, 1981). A second example of normal tissue involvement has been documented with the R-enantiomer of RB 6145, a less emetic bromo-ethylamino precursor of RSU 1069 (Adams and Stratford, 1994). Irreversible retinal degeneration (retinopathy) was reported in murine studies *in vivo*, and consequently the preclinical development of this compound was halted (Parker *et al.*, 1996). Hypoxia is a distinctive feature of retinal tissue that is particularly prominent during low-light vision or the resting state. Thus when considering quinones - not as a bioreductive agents (i.e. quinone methide precursors), but as "trigger" units for the release of diffusible cytotoxins, their dependence on severe hypoxia for appreciable reductive metabolism might, in principle, prove to be an advantageous biochemical property.

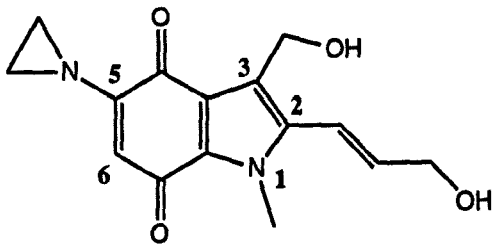
6.1.17 Theoretical considerations in the design of quinone-triggered hypoxia-selective prodrugs of diffusible cytotoxins.

To date, the indoloquinone nucleus has not been described as a trigger mechanism for the selective release of a covalently attached, but independent, cytotoxic unit. In order to usefully apply quinones as hypoxia-dependent triggers, the compounds by definition must not be significant substrates for oxygen-insensitive reductases, in particular DTD. The rational

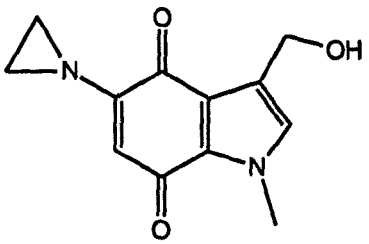
design of the trigger-linker-effector based structures must also allow the reduction of the quinone ("trigger") moiety to facilitate a favourable redistribution of the intramolecular electron density ("linker"), promoting the kinetically efficient activation or release of a latent cytotoxin ("effector"). Additionally, the released "effector" must readily diffuse across cell membrane barriers, be insensitive to oxygen, and (perhaps) more potent than the reduced quinone "trigger" itself.

The indoloquinone structure of EO9 will behave as a trigger molecule, since one-electron reduction will initiate a movement of electrons to evict a leaving group, and in doing so will generate a drug-radical which can alkylate DNA (Oostveen and Speckamp, 1987). EO9 has three distinct functional groups:- the aziridinyl at the C-5 position of the indole ring, a hydroxypropenyl at the 2-position and a C-3 position hydroxymethyl functionality.

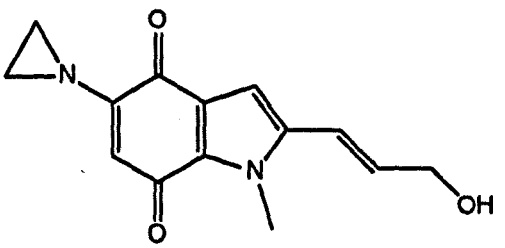
Figure 12: Two closely related analogues of EO9 lacking either the C-2 or C-3 functionality.



EO9



Analogue 2



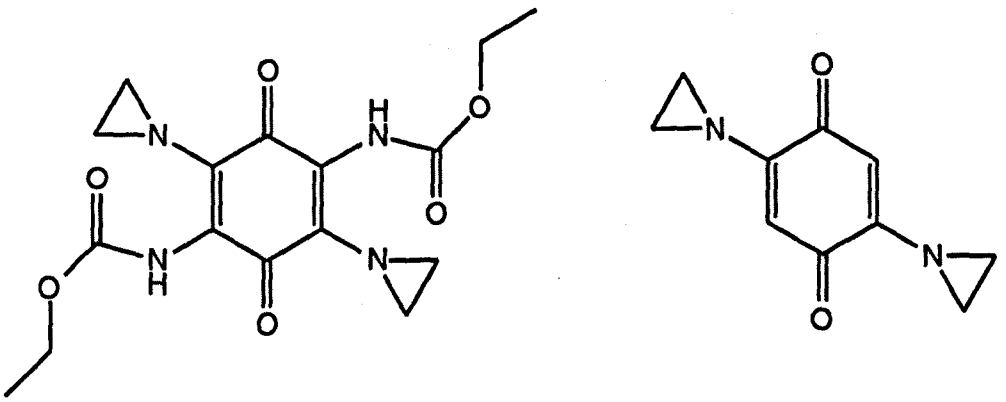
Analogue 3

In order to establish the relative importance of the C-2 and C-3 positions in facilitating $1e^-$-dependent Michael-type elimination processes, the P450R overexpressing MDA 231 clones were utilised to evaluate the oxic and hypoxic cytotoxicity of two EO9 analogues 2 & 3. These analogues lacked either the C-2 or C-3 arm, respectively, allowing the relative

contributions of these groups to both aerobic and hypoxic toxicity to be assessed with respect to one-electron activation.

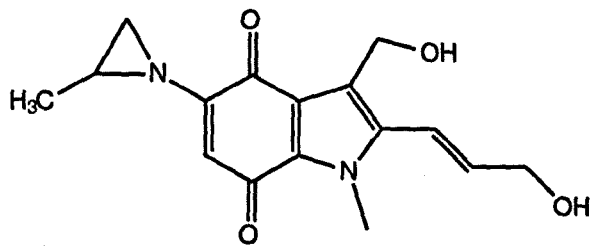
Although the C-5 aziridinyl group contributes significantly to the aerobic potency of EO9, its activity may be enhanced following quinone reduction. As the predicted pK of the aziridine on the semiquinone would be considerably higher than that of the parent quinone, at physiological pH the semiquinone should be more reactive (Butler *et al.*, 1996). This mechanism is exemplified by studies of the reductive activation of the bis-aziridinylbenzoquinones, DZQ and AQZ, where reduction of the quinone function activates attached aziridine groups by initiating electron release and subsequent protonation of the aziridine ring. This allows ring opening, facilitating the subsequent alkylation of nucleophiles (Ross *et al.*, 1990; Ally *et al.*, 1994). Alternatively, under acidic conditions protonation can directly yield an aziridinium ion, which encourages ring opening, releasing the strain energy of the aziridine ring to form a carbonium ion intermediate (Mossoba *et al.*, 1985).

Figure 13: Examples of bis-aziridinylbenzoquinones.

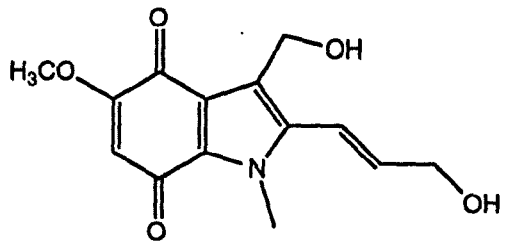


In order to assess this possibility with respect to EO9, two further analogues with modifications at the C-5 position were evaluated. Either the reactivity of the aziridine was moderated by addition of an electron-donating 2'-methyl group (EO8), or it was replaced with a non-alkylating methoxy group (EO7) (Bailey *et al.*, 1992; Naylor *et al.*, 1997; Jaffar *et al.*, 1998a). The relative potency and hypoxic/oxic differential of EO8 and EO7 were tested against the Rd-42 clone.

Figure 14: Two analogues of EO9 with modified C-5 funtional groups.



EO8



EO7

6.1.18 Summary.

This chapter describes the testing of the nitro-triggered prodrugs RSU 1069, CB 1954 and SN 23862, as oxygen-sensitive substrates for the microsomal enzyme P450R. The potency of the bystander effect is also assessed in co-culture experiments *in vitro*. A series of analogues of the indoloquinone EO9 are also evaluated with respect to P450R bioactivation *in vitro*, in order to further the understanding of the design of indoloquinones as potential trigger mechanisms for novel prodrug development. This may allow the rational enzyme-directed design of novel indoloquinone-triggered prodrugs linked to diffusible cytotoxic effectors.

6.2 Methods.

Materials. RSU 1069, CB 1954, SN 23862, EO9 and its four analogues were synthesized in house according to published methods (Adams *et al.*, 1984; Cobb *et al.*, 1969; Palmer *et al.*, 1995; Naylor *et al.*, 1997; Jaffar *et al.*, 1998a). MMC was purchased from Sigma. Porfiromycin was a gift from Dr. Rockwell. All other materials were of tissue culture grade and the highest purity commercially available.

Prodrug sensitivities. Dose-response curves were determined under both aerobic and hypoxic conditions, and IC_{50} values independently collated as previously described (chapter

2). All exposures were 3hr unless otherwise stated. Hypoxic cytotoxicity ratios (HCRs) were calculated as the ratio of drug concentration required to reduce cell growth by 50% under oxic *vs.* hypoxic conditions. Drug sensitivities were assessed in the parental MDA 231 and P450R overexpressing clonal cell lines as described in chapter 3.

Evaluation of "bystander" effects. The P450 reductase overexpressing clone (Rd-42) was co-cultured with the parental MDA-231 cells in 96-well format @ $5x10^3$ cells/well, in increasing proportions (10-50%) to assess the potential for a distal "bystander" killing effect. Modulation of IC_{50} was examined.

Additionally, parental cells were mixed with either 10%, 25% or 50% of Rd-42 cells and seeded at a final density of $5x10^5$ cells/well (i.e. confluent monolayers), allowed to settle overnight, and then exposed to a concentration range of the test prodrug for 24hr under hypoxic conditions. Cells were washed free of drug and 200 µl of 1x trypsin/EDTA (Gibco BRL) was added for 10 min. Plates were agitated vigorously to ensure a single cell suspension, and the plates were poured out and blotted on a pad of sterile tissue. This removed all but a thin film of liquid containing residual cells, so that only ≈10% of the original cell population remained behind. Plates were refed with fresh media and cells were allowed to proliferate for a further 96hr. This re-spreadingassay allowed the IC_{50} profiles to reflect only the growth of the target (low-P450R) cells. Modulation of IC_{50} was examined at the different ratios of target and activator cells, and when pooled, allowed the relationship between drug sensitivity and target/activator cell proportions to be interrelated.]From this, a value of the efficiency of bystander cell killing could be interpolated.

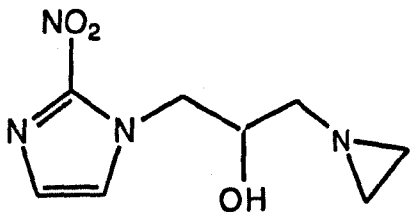
In a further experiment, parental MDA-231 "marker" cells were seeded @ $1x10^4$ cells/well (24 well plates), and either $2x10^6$ Rd-42 cells (activator cells) or $2x10^6$ parental cells (control cells) were seeded into a porous membrane co-culture insert (0.2 µm pores). 0.5 ml of complete media was added to connect the two cell culture compartments in the media phase. Prodrug (100 µl) was added to the insert well, at the ≈ IC_{50} concentration for the Rd-42 cell line, and incubated for 3hr under hypoxia. Following exposure, "marker" cells were washed free of drug and allowed to proliferate for 96hr.

All exposures were carried out in a catalyst-induced anoxic chamber, and all plastic and media was equilibrated with the anoxic enviroment for > 24hr prior to experimentation.

6.3 Results.

Sensitivity of P450R overexpressing cell lines to RSU 1069 under aerobic and hypoxic conditions *in vitro*. Analysis of the sensitivity of four of the P450R-transfected MDA 231 clones to RSU 1069 was conducted, and IC_{50} values were determined as previously described. As summarised in table 1, the data demonstrated a clear relationship between elevated P450 reductase activity and increased sensitivity to RSU 1069 under 3h hypoxic (slope = -1.61 ±0.22; P=0.018). However no relationship was apparent for the 3h aerobic (slope = -14.8 ±9.2; P=0.11) exposure conditions (Figure 15). The line of best fit for each relationship clearly demonstrated that elevations in P450R expression conferred hypoxia-specific modulation of RSU 1069 prodrug sensitivity.

Table 1: Relationship of acute aerobic and hypoxic RSU 1069 toxicity with increasing P450 reductase activity in the human breast adenocarcinoma cell line MDA 231.



Clonal cell line	RSU 1069 (IC_{50} μM ± s.d.)		Hypoxic cytotoxicity ratios
	Aerobic	Hypoxic	(HCRs)
Parental	280 ± 62	176 ± 39	1.6
Rd-06	289 ± 47	239 ± 44	1.2
Rd-16	269 ± 66	38.8 ± 10.8	6.9
Rd-22	258 ± 111	19.0 ± 5.3	13.5
Rd-42	196 ± 47	20.3 ± 7.0	9.7
wt/Rd42 fold-sensitization	1.4	9.3	

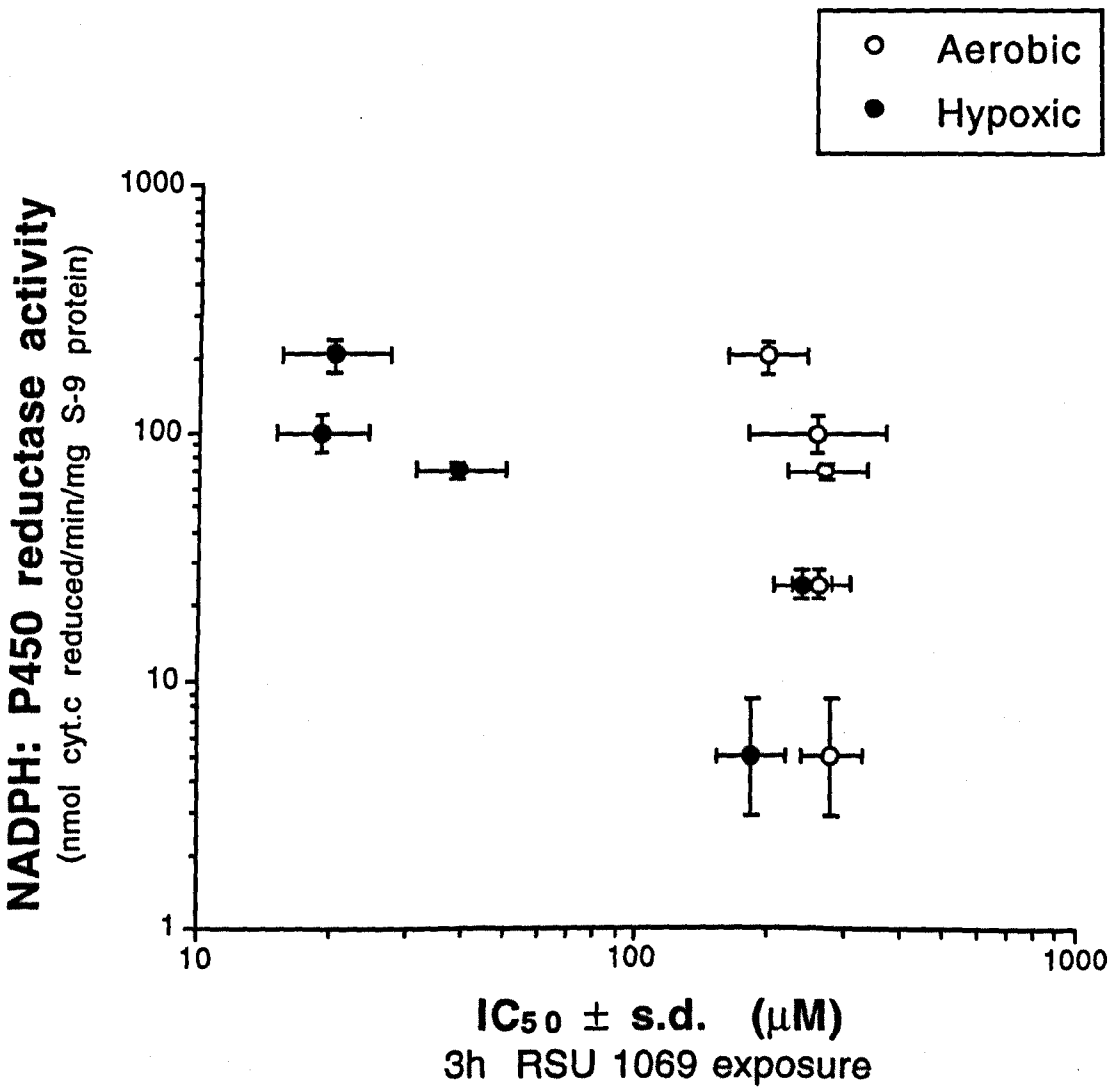
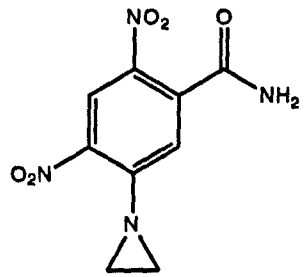
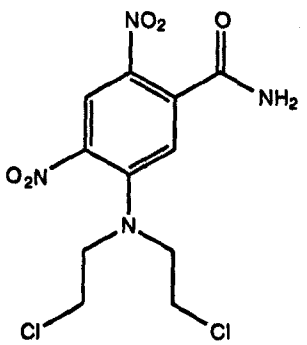


Figure 15: Relationship between acute (3h) oxic and hypoxic sensitivity to the 2-nitroimidazole RSU 1069 and intracellular NADPH:cytochrome P450 reductase (P450R) activity in the parental MDA-231 breast cell line and four P450R-overexpressing clonal lines.

Hypoxic and aerobic sensitivity of Clone Rd-42 to CB 1954 and SN 23862 *in vitro*. Due to limited material availability, CB 1954 and its nitrogen mustard analogue SN 23862 were screened against two cell lines; the parental MDA 231 and clone Rd-42. IC_{50} values were determined, from which HCRs were calculated, as before.

Table 2: Relative sensitivity of MDA 231wt and Rd-42 cell lines to the 2,4-dinitrobenzamide, CB 1954, and its nitrogen mustard analogue SN 23862 under oxic and hypoxic conditions.





Cell line	CB 1954 (IC_{50} µM ± s.d.)			SN 23862 (IC_{50} µM ± s.d.)		
	Aerobic	Hypoxic	HCRs	Aerobic	Hypoxic	HCRs
Parental	780 ±45	90 ±11	8.7	730 ±61	780 ±88	0.93
Rd-42	350 ±38	9 ±0.6	39	630 ±58	80 ±10	8.2
wt/Rd42 fold-sensitization	2.2	10.0		1.1	9.8	

Although it is not possible to compare directly the relative stoichiometry of the relationships between increasing P450R activity and prodrug sensitivities (IC_{50}), with respect to RSU 1069 (i.e. the slope of the line of best fit), it is clear that a similar oxic/hypoxic asymmetry emerges. Very limited aerobic sensitization occurs as a consequence of elevated P450R activity, with enhanced prodrug sensitivity being strongly dependent upon the hypoxic environment. The pattern of sensitivity between the two dinitrobenzamide analogues is significantly different. For the parental cell line, potency is equivalent under aerobic exposure conditions, but only CB 1954 produces any differential sensitivity under hypoxia (figure 16). This is unusual, since the majority of cell lines exhibit superior HCRs for SN 23862 when compared to CB 1954 (Palmer *et al.*, 1992). However the hypoxia-dependent sensitization

observed in the Rd-42 cell line is broadly similar, with a 42-fold increase in P450R activity producing a similar ≈ 10-fold decrease in IC_{50} value for both prodrugs.

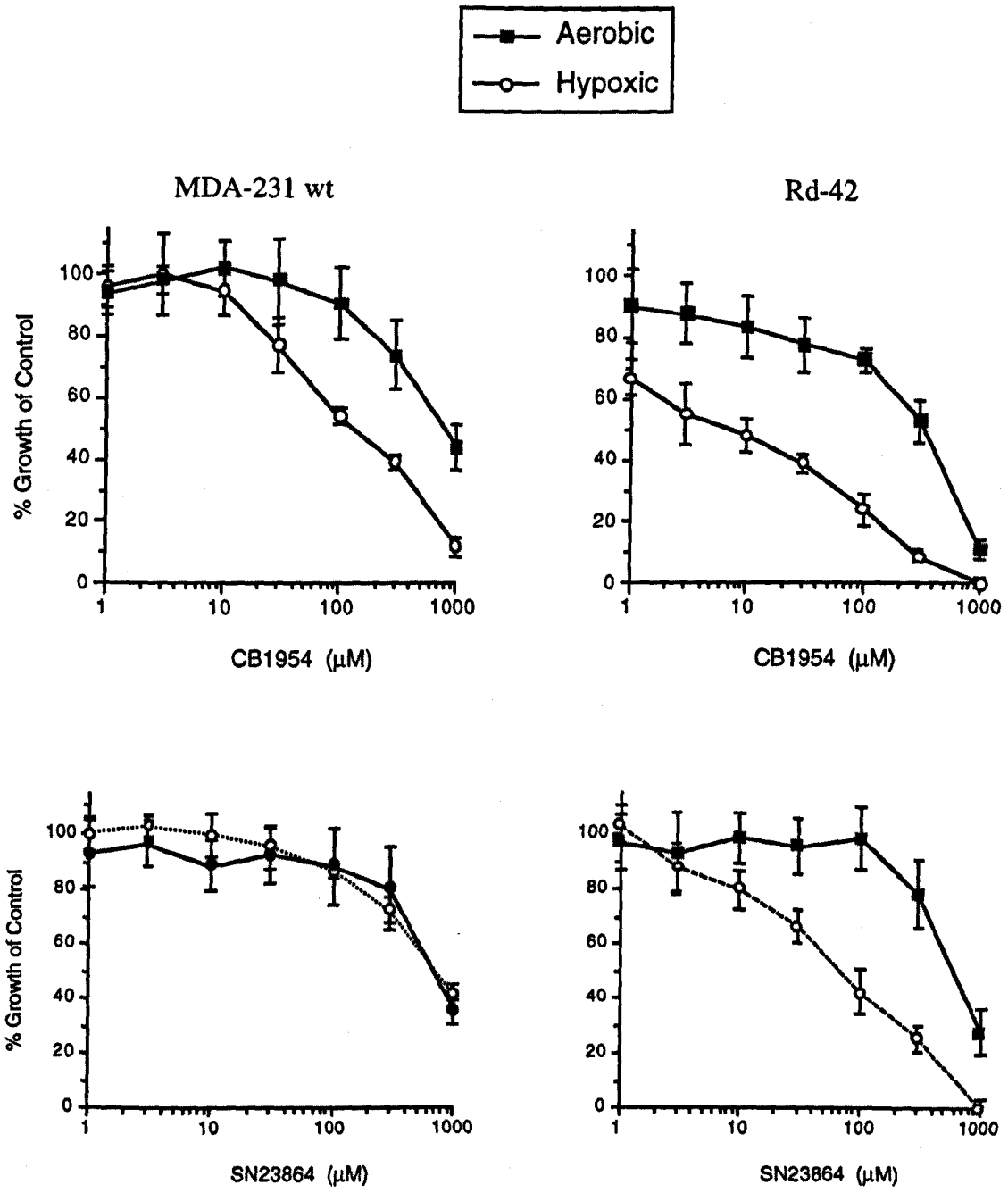
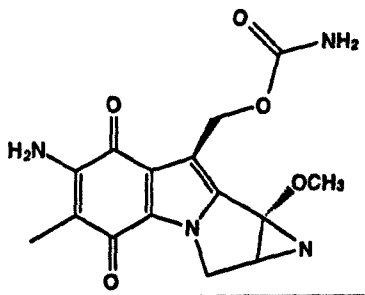


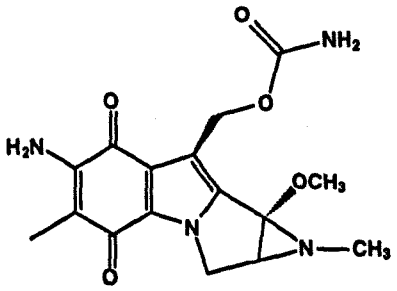
Figure 16: Representative dose response curves for acute (3h) hypoxic and aerobic exposure to the aziridinyl 2,4-dinitrobenzamide CB 1954 and its nitrogen mustard analogue SN 23862 in the parental MDA-231 and its P450R over-expressing Rd-42 clonal cell line.

Since SN 23862 sensitivity is not significantly influenced by P450R under aerobic conditions, its activation is more specific for oxygen-inhibitable reduction by P450R than CB 1954, i.e. the HCRs for CB 1954 and SN 23862 were enhanced 4.5- and 8.8-fold by the presence of elevated P450R activity, respectively. This specificity, coupled with the fact that SN 23862 is not a substrate for human DT-diaphorase (Palmer *et al.*, 1992), suggests that this agent (or a more soluble analogue) might be an appropriateprodrug to employ in an O_2 - sensitive GDEPT strategy. In contrast, CB 1954 is a (poor) substrate for DT-diaphorase (Boland *et al.*, 1991; Knox *et al.*, 1993; Beall *et al.*, 1994; Chen *et al.*, 1995c), and consequently the oxygen-inhibited reduction of CB 1954 might be superseded by direct two-electron metabolism. This difference in substrate specificity is of little relevance in the MDA 231 cell line model, since cytosolic DT-diaphorase activity is barely detectable (chapter 2).

Confirmation of the role of P450R in the bioactivation of mitomycin C and Porfiromycin under aerobic and hypoxic conditions. Due to limited material availability, mitomycin C (MMC) and its methyl-aziridine analogue, porfiromycin (POR), were screened against two cell lines; the parental MDA 231 and clone Rd-53. IC_{50} values were determined, from which HCRs were calculated.

Table 3: Relative sensitivity of MDA 231wt and Rd-53 cell lines to mitomycin C (MMC) and its methyl-aziridine analogue, porfiromycin (POR), under oxic and hypoxic conditions.





Cell line	Mitomycin C (IC_{50} μM ± s.d.)			Porfiromycin (IC_{50} μM ± s.d.)		
	Aerobic	Hypoxic	HCRs	Aerobic	Hypoxic	HCRs
Parental	10.6	3.7	2.8	1100	70	15.7
Rd-53	2.5	0.7	3.6	180	9.3	19.4
wt/Rd53 fold-sensitization	4.2	5.3		6.1	7.5	

In contrast to the nitro-prodrugs, elevated P450R activity conferred nearly equal sensitization to MMC and POR under both aerobic and hypoxic exposure conditions. Some selectivity was apparent, since the HCR values in the Rd-53 cell line were modestly increased for both MMC and POR. This difference in oxygen-sensitivity of activation by P450R when compared to the nitro-containing agents is unlikely to represent any fundamental distinction in the mechanism of reduction by FMN semiquinone of P450R, since the lack of a distinct substrate binding site precludes the possibility of any specific structure-activity relationships. Rather it most likely represents a distinction in the chemistry of the reduced prodrugs and their equilibria with molecular oxygen and their hydroquinone forms. It is possible that eviction of the C-9 methoxy group which preceeds intramolecular rearrangement, is sufficiently rapid to compete with the backoxidation of the semiquinone radical species.

Alternatively, the rate of disproportionation of the $Q^{\bullet-}$ radicals to the oxidation-insensitive QH_2 may be sufficiently rapid in the localised enzyme enviroment, that oxygen-selective activation is lost. This would result in the expression of cytotoxicity irrespective of the oxygen status of the activating cells. Less likely, changes in the rate of ROS generation by futile cycling might be nearly as cytotoxic as that derived from the enhanced activation of the indole quinones themselves under anaerobic conditions, particularly if redox metal ion availaility was good within close proximity to DNA. The observation that the differential toxicity of POR is significantly greater than that of MMC might also suggest that the intrinsic reactivity of the aziridine group is, in part, responsible for the P450R-dependent prodrug sensitisation in both the presence and absence of molecular oxygen. However, the relatively poor oxygen-sensitivity of mitomycin prodrug one-electron bioactivation, and the complex nature of structural rearrangement that occurs upon reduction, when coupled with the limited synthetic chemistry design options for novel analogue derivatives does not make this particular type of bioreductive prodrug an attractive model for the design of oxygen-sensitive trigger mechanisms.

Hypoxic and aerobic sensitivity of P450R overexpressing cell lines to EO9 and two closely related analogues lacking functionality in the C-2 and C-3 positions *in vitro*. Analysis of the sensitivity of four of the P450R-transfected MDA 231 clones to EO9 was conducted, and IC_{50} values were determined. In contrast to RSU 1069, there was a clear relationship between P450 reductase activity and increased sensitivity to EO9 under both 3h hypoxic (slope = -1.87 ±0.28; $P<10^{-6}$) and 3h aerobic (slope = -7.10 ±3.0;

P=0.022) exposure conditions (Figure 17), although, unlike the mitomycin antibiotics, the aerobic sensitization was very modest. Table 4 summarises the data set.

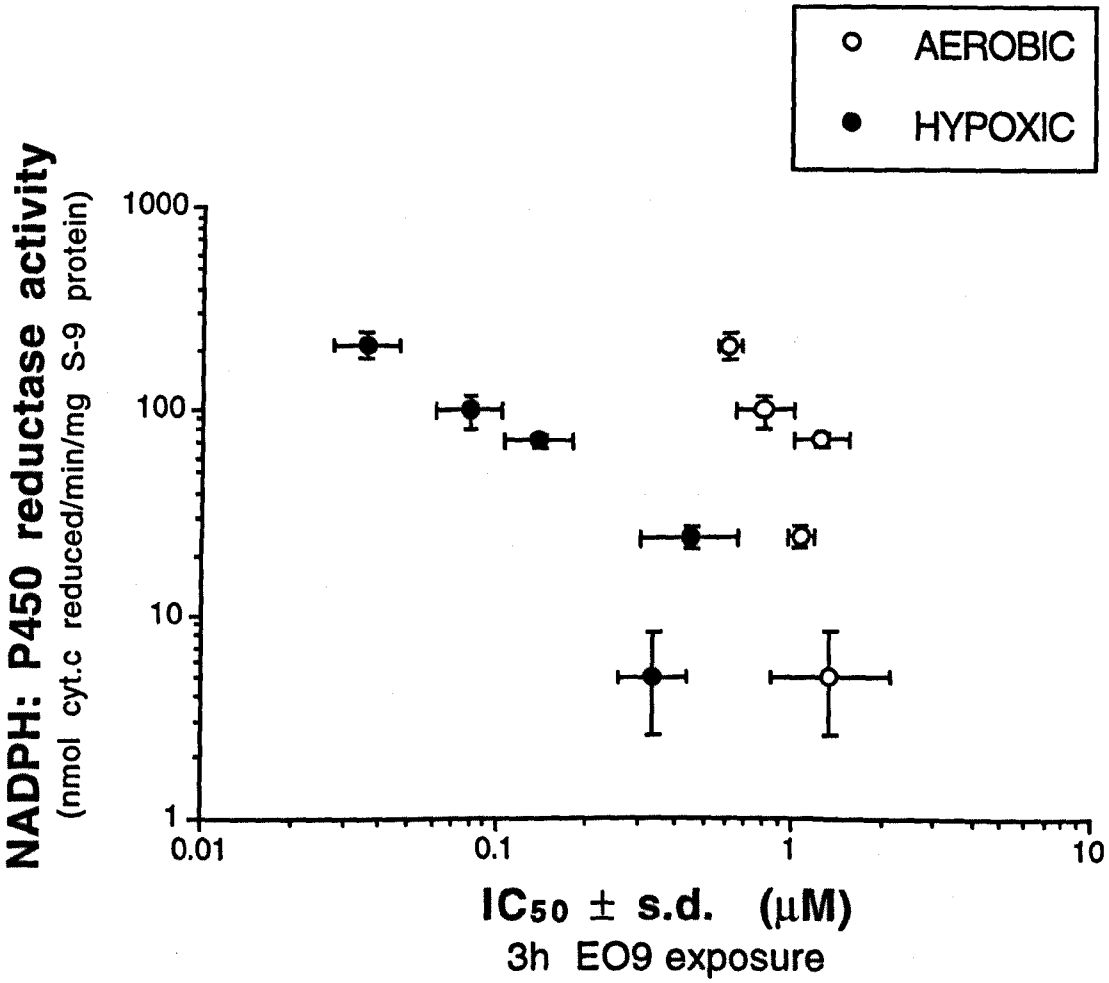
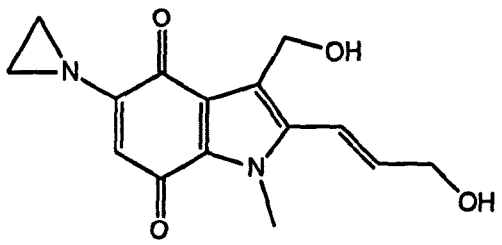


Figure 17: Relationship between acute (3h) oxic and hypoxic sensitivity to the indoloquinone EO9 and intracellular NADPH:cytochrome P450 reductase (P450R) activity in the parental MDA-231 breast cell line and four P450R-overexpressing clonal lines.

Table 4: Relationship of acute aerobic and hypoxic EO9 toxicity with increasing P450 reductase activity in the human breast adenocarcinoma cell line MDA 231.



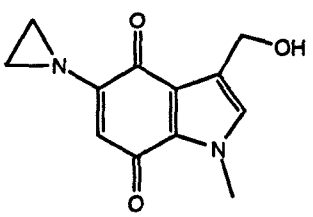
Clonal cell line	EO9 (IC_{50} μM ± s.d.)		Hypoxic cytotoxicity ratios
	Aerobic	Hypoxic	HCRs
Parental	1.34 ± 0.92	0.338 ± 0.15	4.0
Rd-06	1.05 ± 0.29	0.449 ± 0.29	2.3
Rd-16	1.25 ± 0.43	0.136 ± 0.08	9.2
Rd-22	0.75 ± 0.35	0.079 ± 0.05	9.5
Rd-42	0.61 ± 0.06	0.036 ± 0.02	16.9
wt/Rd42 fold-sensitization	2.17	9.39	

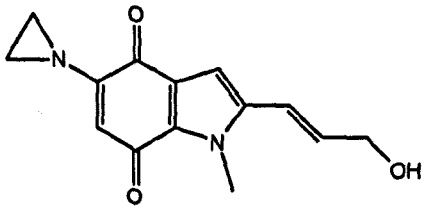
The requirement for hypoxia in the P450R-mediated enhancement of EO9 toxicity was clearly apparent. The extent of this phenomenon was comparable to that observed with the nitro prodrugs. This is seen in the increasing HCR values with increasing P450R activity. However, while these differential toxicities for EO9 were broadly similar to those of the nitro-prodrugs, the absolute (molar) potency of EO9 was approximately 500 to 1500 -fold greater under these exposure conditions. This potency may, in part, be a reflection of the rapid rates of indoloquinone disproportionation (relative to nitroarenes), but might also arise through fundamental differences in the nature of the DNA-drug lesions, such as the capacity for hydrogen bonding between the DNA bases which can, in some instances, favour the formation of crosslinks as opposed to single alkylations (Hartley *et al.*, 1991; Mayalarp *et al.*, 1996).

For the two closely related analogues of EO9, lacking either the C-2 or C-3 functionality, the tight relationship between P450R activity and hypoxic and aerobic cytotoxicity was still clearly apparent (table 5). However, compared to EO9, both analogues had 5 to 20 -fold lower IC_{50} values, indicating that both these functional leaving groups

contribute, in part, to the potency of EO9. Yet the pattern of cytotoxicity differed from that of EO9, with respect to the relationship with P450R activity and its relative influence on either the oxic or hypoxic sensitization (figures 18 and 19).

Table 5: Relationship of acute aerobic and hypoxic toxicity for analogue 2 and 3, with increasing P450 reductase activity in the human breast adenocarcinoma cell line MDA 231.

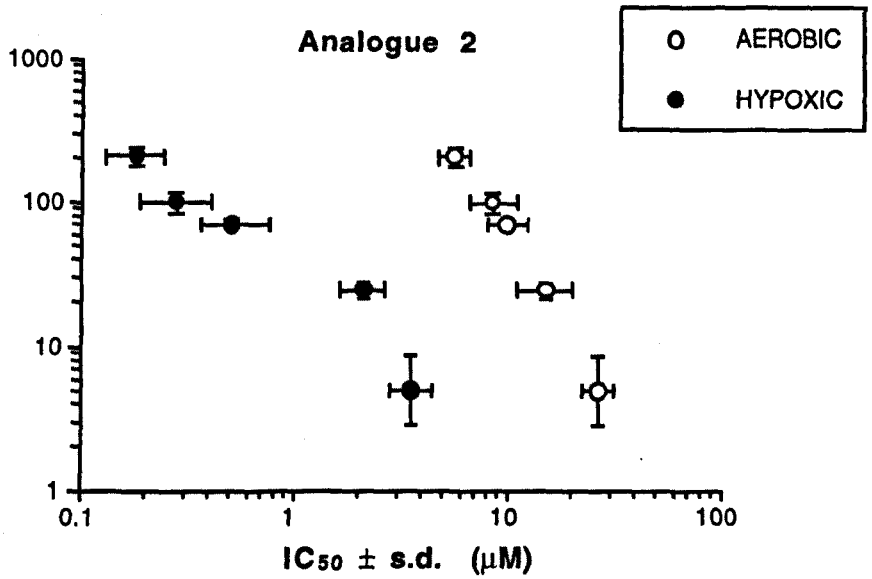


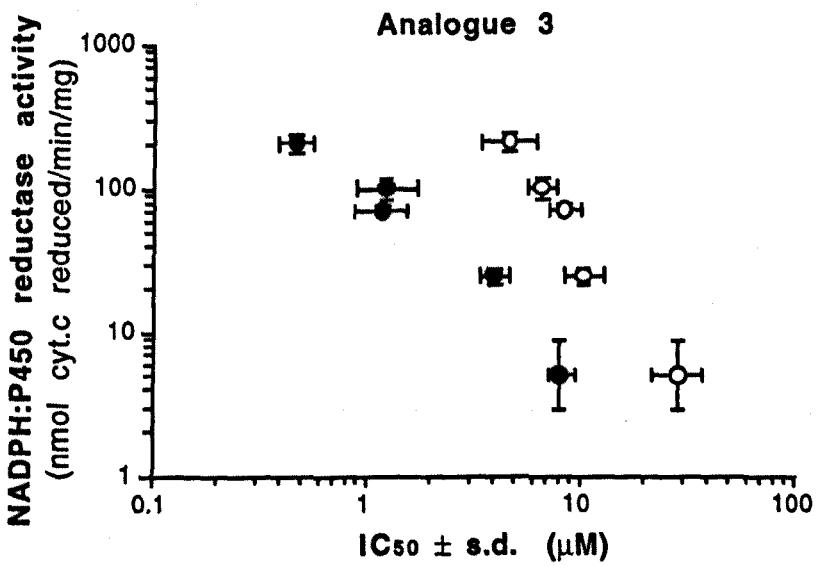


Cell line	Analogue 2 (IC_{50} µM ± s.d.)			Analogue 3 (IC_{50} µM ± s.d.)		
	Aerobic	Hypoxic	HCRs	Aerobic	Hypoxic	HCRs
Parental	26.5 ±3.0	3.54 ±0.90	7.5	28.7 ±9.1	8.16 ±0.15	3.5
Rd-06	15.1 ±5.2	2.10 ±0.55	7.2	10.5 ±4.2	4.01 ±0.29	2.6
Rd-16	9.9 ±2.4	0.51 ±0.41	19.4	8.4 ±1.8	1.18 ±0.08	7.1
Rd-22	8.5 ±2.6	0.28 ±0.20	30.4	6.6 ±1.1	1.24 ±0.05	5.3
Rd-42	5.6 ±1.1	0.18 ±0.08	31.1	4.7 ±1.6	0.46 ±0.02	10.1
wt/Rd42 fold-sensitization	4.7	19.7		6.2	17.7	

For analogue 2, lacking the C-2 hydroxypropenyl arm, the relationship between hypoxic IC_{50} and P450R activity was significantly tighter (slope = -1.15 ± 0.15; $P< 10^{-6}$) than seen for EO9 (slope = -1.87), but, unlike EO9, this was also accompanied by a rise in the aerobic sensitization by P450R (slope = -2.54 ± 0.30; $P< 10^{-6}$) (figure 18). Similarly analogue 3, lacking the hydroxymethyl at the 3-position, showed a closer relationship with P450R under hypoxia (slope = -1.36 ± 0.21; P= 0.008) than EO9, and again this was paralleled by a rise in aerobic cytotoxicity (slope = -2.23 ± 0.25; $P< 10^{-6}$) (figure 19). While both analogues were of equivalent potency in the presence of oxygen, analogue 2 was more cytotoxic than analogue 3 under hypoxic conditions, suggesting that the contribution of the hydroxymethyl group at C-3 was greater than that of the leaving group at C-2 under these conditions. This perhaps reflects a difference in the energetic requirements for the movement

of electrons necessary to evict the respective C-2 or C-3 leaving groups and generate the activated alkylating species.

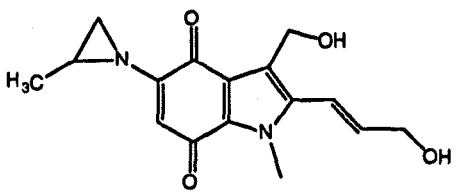


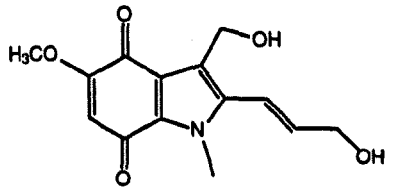


Figures 18 & 19: Comparative cytotoxicity (IC_{50}) of the EO9 analogues 2 and 3, lacking functionality in either the C-3 or C-2 positions of the indole ring, respectively: relationship to P450R activity in the MDA 231 cell line under aerobic and hypoxic conditions *in vitro*.

Hypoxic and aerobic sensitivity of Clone Rd-42 to C-5 aziridinyl-modified EO9 analogues; EO8 and EO7. Moderation of the C-5 aririidine reactivity by introduction of methyl group at the 2-position of the aziridinyl-ring, to produce the analogue EO8, resulted in the lowering of the aerobic toxicity for both cell lines, without loss of hypoxic potency. Consequently the differential toxicity (HCR) of EO8 is superior to that of EO9 in both the parental and Rd-42 cell lines (table 6). Overexpression of P450R resulted in the hypoxia-selective enhancement of EO8 cytotoxicity, suggesting that reduction of the quinone initiates a redistribution of electron density that activates the methyl-aziridinyl functionality. This activation of the C-5 position upon one-electron reduction was more pronounced than for EO9, where the more reactive aziridinyl moiety was not as oxygen-inhibitable. This difference is unlikely to arise as a consequence of a change in the substrate specificty of EO8 for the two-electron reductase DT-diaphorase since endogenous activity is negligible in this cell line model. The importance of the C-5 aziridinyl group is further demonstrated with the C-5 methoxy analogue EO7, where a 100 to 150-fold loss in potency, relative to EO9, was found under both aerobic and hypoxic exposure conditions. Furthermore, the differential toxicity for EO7 was narrowed in both cell lines, primarily through a loss of the oxygen-inhibitable activation by P450R. This agrees with the analysis of EO8, indicating that the latent reactivity of the C-5 methyl-aziridinyl group contributes to the hypoxia-selectivity of the indoloquinone analogues.

Table 6: Relative sensitivity of MDA 231wt and Rd-42 cell lines to the C-5 2-methylaziridin-1-yl analogue, EO8, and its C-5 methoxy analogue, EO7, under oxic and hypoxic conditions.





Cell line	EO8 (IC_{50} μM ± s.d.)			EO7 (IC_{50} μM ± s.d.)		
	Aerobic	Hypoxic	HCRs	Aerobic	Hypoxic	HCRs
Parental	4.6 ±1.0	0.40 ±0.08	11.5	190 ±29	51.1 ±15	3.7
Rd-42	3.8 ±1.6	0.07 ±0.02	54.3	25.3 ± 21	6.1 ±0.5	4.1
wt/Rd42 fold-sensitization	1.2	5.7		7.5	8.4	

Taken together these data suggests that the aziridinyl group in the C-5 position of the indole ring is the single most important determinant of the absolute potency of EO9, but its reactivity is not strongly influenced by reduction of the quinone. However, modification of this aziridine by addition of a methyl group lowers the intrinsic reactivity of the moiety, and in doing so appears to make it more responsive to the oxygen-sensitive reduction of the quinone. In agreement removal of the C-5 functionality (EO7) lowers the HCR values and results in a major loss of cytotoxic specificity for oxygen-inhibitable one-electron reduction. Consequently P450R was almost as efficient at activating EO7 under aerobic conditions as it was under hypoxia.

Dependence of the hypoxia-dependent "bystander" cell killing effect on cellular density *in vitro*. The dose-response curves generated from 100% parental (target), or 100% Rd-42 (activator) cells allow a measure of cytotoxicity (IC_{50}) to be determined for the three nitro-prodrugs. The ratio between the IC_{50} of these cell lines under hypoxia is the dose modification factor accompanying the elevated activation of the prodrugs. When activator and target cells are mixed, intermediate curves were generated, the positions of which relative to the two extremes, is characteristic of a cross-sensitization phenomenon.The bystander effect of the prodrugs is a composite of the ability of the activated product to diffuse between cells, either through intimate cell-to-cell contact (confluent monolayers) or via the media phase (sparse single-cell culture), and is related to the proportion of activator cells present. For each defined proportion of activator cells a single IC_{50} value is derived. This can then be expressed relative to the two extreme IC_{50} determinations to derive a value for the % of activator cells required to make the target cells experience 50% of their toxicity. Thus the proportion (%) of Rd-42 activator cells required to reduce the parental IC_{50} value for the prodrug to halfway between the IC_{50} for 100% Rd-42 cells and 100% parental cells is termed the "50% transmission efficiency" (TE_{50}). The relative TE_{50} values thus provide a single comparative unit of the bystander killing efficiency for each prodrug. Application of this technique has been described recently in the context of *E. coli* NR-activated CB 1954 bystander quantification (Friedlos *et al.*, 1998).

In the low density co-culture experiments *in vitro* only SN 23862 demonstrated any measurable bystander effect although this was modest (figure 20). RSU 1069 and CB 1954 were both apparently unable to measurably cross-sensitize parental "target" cells under hypoxic conditions. The TE_{50} values calculated were 50, 48 and 39 for RSU 1069, CB 1954 and SN 23862, respectively. Thus in the case of RSU 1069, (TE_{50} = 50), a 50:50 mix of

parental and Rd-42 cells was necessary to shift the IC_{50} value by 50%, indicating the absence of any bystander phenomena.

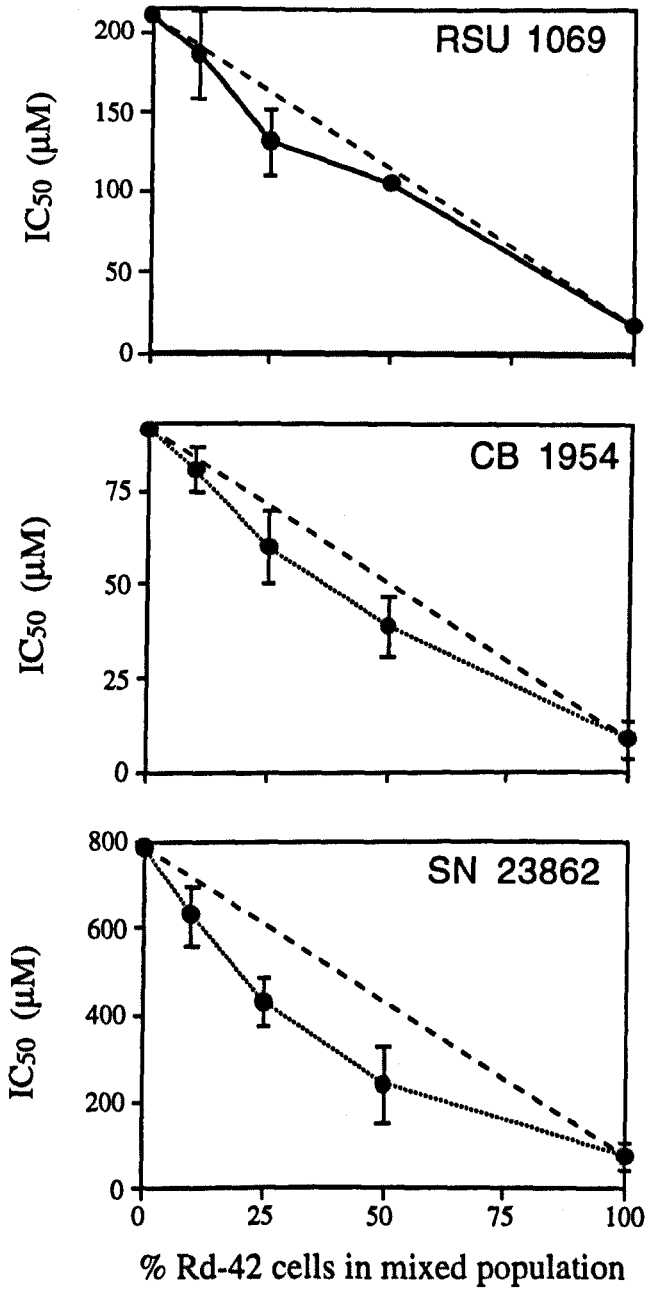


Figure 20: Comparative bystander effects of three nitro-prodrugs in sparse ($5x10^3$ cells/well) mixed populations of "marker" parental MDA 231 cells and hypoxia-dependent "activator" Rd-42 cells overexpressing human P450 reductase *in vitro*. (Broken line indicates predicted plot if no co-sensitization effect occurs).

The ability of SN 23862 to remain sufficiently stable in the media phase following reductive activation, was confirmed in the subsequent Costar™ porous well-insert experiments. Even when the P450R-overexpressing cells were physically separated from the parental "marker" cells, the connection via the media phase was sufficient to allow transfer of prodrug sensitivity (Table 7). Indeed, the excess of Rd-42 activating cells ($2x10^6$) in the porous insert inhibited the growth of the parental cell line slightly more than the Rd-42 cells were inhibited in the absense of any co-culture. Co-incubation with parental cells could not mimic this phenomenon.

Table 7: Evaluation of the hypoxia-dependent "bystander" effect: Qualitative assessment of metabolite toxic transfer from the P450R-overexpressing cell line Rd-42 to the low expressing parental cell line.

Marker cell line:		Parental	Rd-42	Parental	Parental
Co-culture insert cell line:		-	-	Parental	Rd-42
Concn.	Drug	% growth of untreated control			
30 μM	RSU 1069	85	47	87	89
10 μM	CB 1954	88	49	84	78
100 μM	SN 23862	87	46	81	37 *
0.1 μM	EO8	83	48	83	87

* Significantly different from "marker" cells alone (n= 2; P< 0.01).

In a further experiment TE_{50} values were calculated using the confluent monolayer model ($2x10^6$ cells/well) in order to identify the presence of any labile toxic metabolites that might confer a highly localized sensitization of neighbouring cells. In this two-dimensional monolayer model all three nitro-prodrugs were found to produce a hypoxia dependent bystander effect. TE_{50} values of 12.7, 2.2 and 4.9 were extrapolated for RSU 1069, CB 1954 and SN 23862, respectively (figure 21).

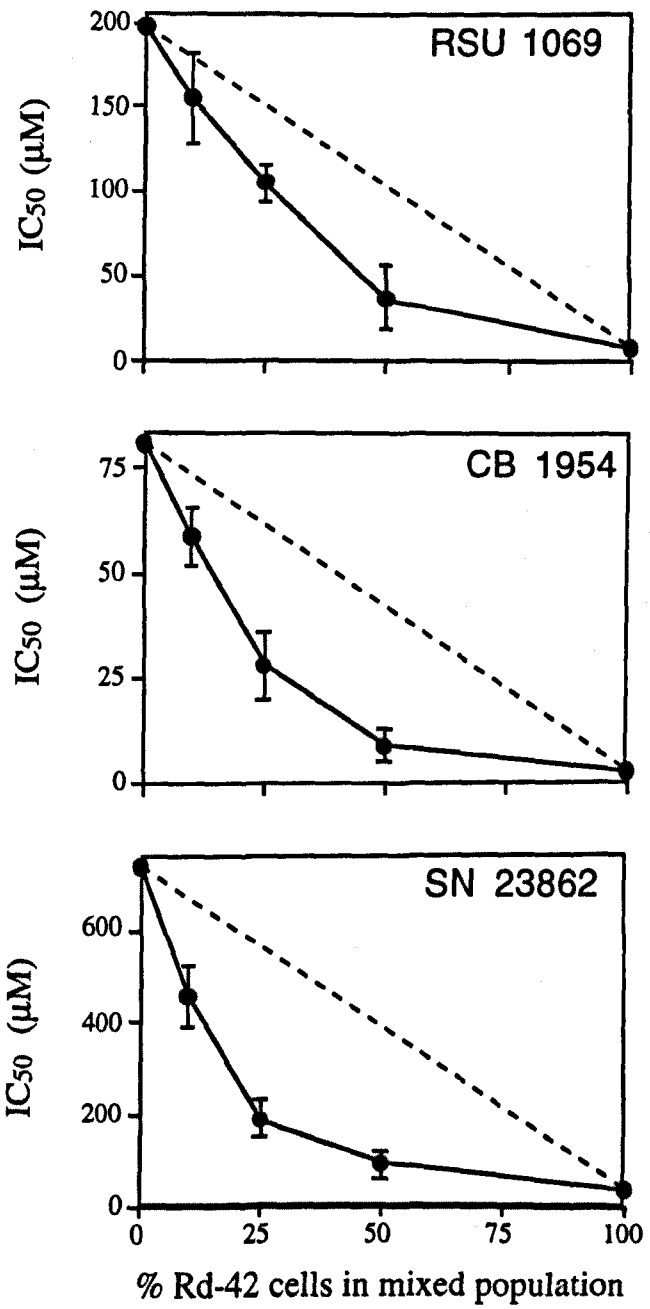


Figure 21: Comparative bystander effects of three nitro-prodrugs in confluent ($2x10^6$ cells/well) mixed populations of "marker" parental MDA 231 cells and hypoxia-dependent "activator" Rd-42 cells overexpressing human P450 reductase *in vitro*. (Broken line indicates predicted plot if no co-sensitization effect occurs).

Thus, in this particular *in vitro* model CB 1954 provided a superior bystander effect to that of SN 23862, despite the earlier observations that only SN 23862 could facilitate cross-sensitization in the non-contact experiments. This suggests that the reduction product(s) of SN 23862 are sufficiently stable to diffuse large distances in an aqueous enviroment, but by inference is too non-reactive to retain the effect locally in a two-dimensional bystander model. Consequently much of the activated drug is dissipating into the third-dimension, the large volume of media above the cells, limiting the potency of the cross-sensitization effect. CB 1954, in contrast, is significantly more reactive, such that the drug exerts its effects in a more localised fashion. As a result the mono-layer model suggests the presence of a superior "transmission efficiency", which is not strictly true. In a three-dimensional tumour mass, the capacity to diffuse beyond the localised activation site may be an advantage, if the intention is to exploit the foci of acute hypoxia that occur as a consequence of vascular occlusion, although excessive diffusion would be undesirable. Thus the use of a three-dimensional *in vitro* model, such as spheroids, or multi-cellular co-culturing techniques (Cowan *et al.*, 1996), would probably provide a more accurate representation of the utility of these prodrugs as hypoxia-activated diffusible cytotoxins.

6.4 Discussion.

6.4.1 Comparative sensitivity of the MDA 231 cell line to chemotherapeutic agents.

The MDA 231 cell line was anomalous in its poor response to RSU 1069 under hypoxic exposure conditions. This is reflected in the very low HCR value of 1.6, which is the lowest value reported for any cell line evaluated with RSU 1069 (Patterson *et al.*, 1997). This appeared to manifest in the limited differentials to all the other hypoxic cytotoxins evaluated in this study. For example, the differential toxicity for EO8 was 11.5-fold in the parental cell line, but identical experiments in the rodent V79 cell line, revealed a HCR value in excess of 5000-fold (Jaffar *et al.*, 1998a). This is the highest value (by a factor of 100) for differential toxicity ever reported for any bioreductive indoloquinone (for comparison with other HCRs for indoloquinones in V79 cells see:- Moody *et al.*, 1994; Cotterill *et al.*, 1994), and is in fact the largest published value for the oxic/hypoxic differential of any bioreductive drug to date.

This impressive differential is also seen, to a lesser degree, in human cell lines, where values upto 500-fold are seen (unpublished data).

A possible explanation for the underlying mechanism of tolerance to these alkylating bioreductives can be found in the pattern of atypical resistance displays towards unrelated chemical crosslinking agents such as cis-Platin and chloroambucil (Houlbrook *et al.*, 1994). This suggests that the MDA 231 cell line is relatively competent at recognising and repairing DNA-DNA crosslink adducts, and this resistance is independent of the enzymology of the cells. However the low reductive enzyme status of the parental cell line (chapter 2) will also contribute to the lack of hypoxic sensitivity to bioreductive alkylating agents, as indicated by the trend of increasing HCRs for all agents with increasing P450R expression.

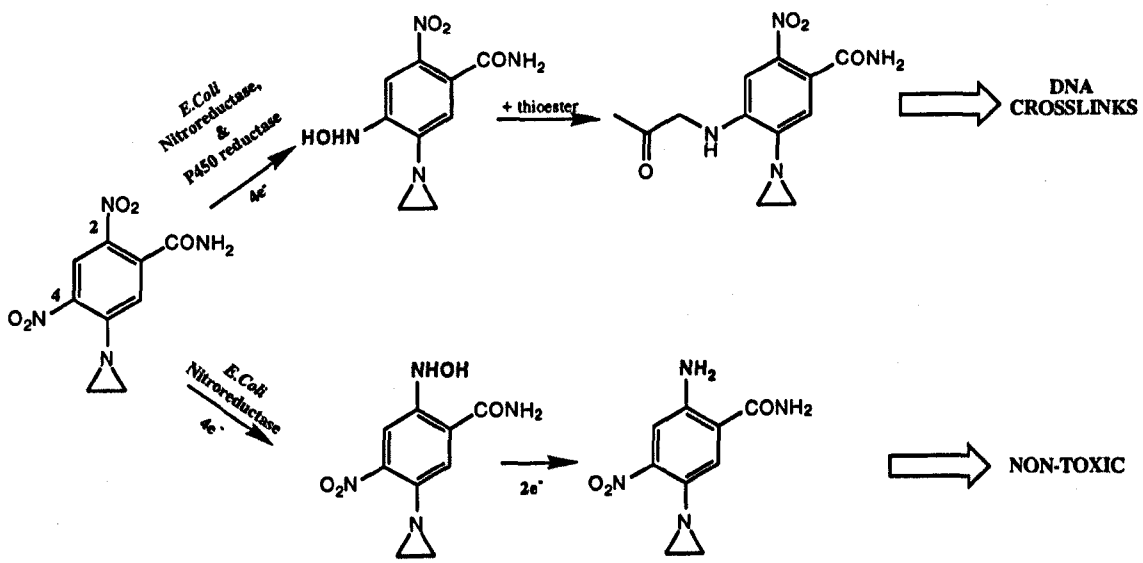
6.4.2 Comparative utility of the nitro-prodrugs.

All three nitro-prodrugs were oxygen-inhibited substrates for P450R, indicating they can act as anaerobic electron acceptors from the semiquinone flavin. The fold-sensitization under hypoxia was approximately equivalent for each agent in the Rd-42 cell line. Only CB 1954 cytotoxicity was significantly modulated by elevated P450R activity under aerobic conditions (2.2-fold). CB 1954 was also unique in its capacity to produce a significant HCR in the parental cell line. The result might imply that CB 1954 is a substrate for another endogenous one-electron reductase(s), while SN 23862 and RSU 1069 are not. The comparable sensitization of Rd-42 to the three nitro-triggered prodrugs is consistent with their similar one-electron reduction potentials (-398, -385 and -371 mV for RSU 1069, CB 1954 and SN 23862, respectively), which has been demonstrated to be the primary determinant of the rate of metabolism by P450R (Butler and Hoey, 1993b). However, one further possibility that can not be ruled out, is that the nature of the DNA-DNA crosslinks differs between these compounds in a complex and yet undefined manner.

The differing patterns of sensitivity between the two 2,4-dinitrobenzamide analogues, SN 23862 and CB 1954, may be related to the stoichiometry of 2- vs. 4-nitro reduction by P450R, and its influence on DNA damage. For CB 1954, the 4-hydroxylamine reduction product is the potent cytotoxic agent capable of producing DNA-DNA interstrand crosslinks in cells, while the 2-hydroxylamine species is much less cytotoxic, and only strand breaks have been observed (Knox *et al.*, 1988). Under hypoxia, P450R preferentially reduces the 4-nitro

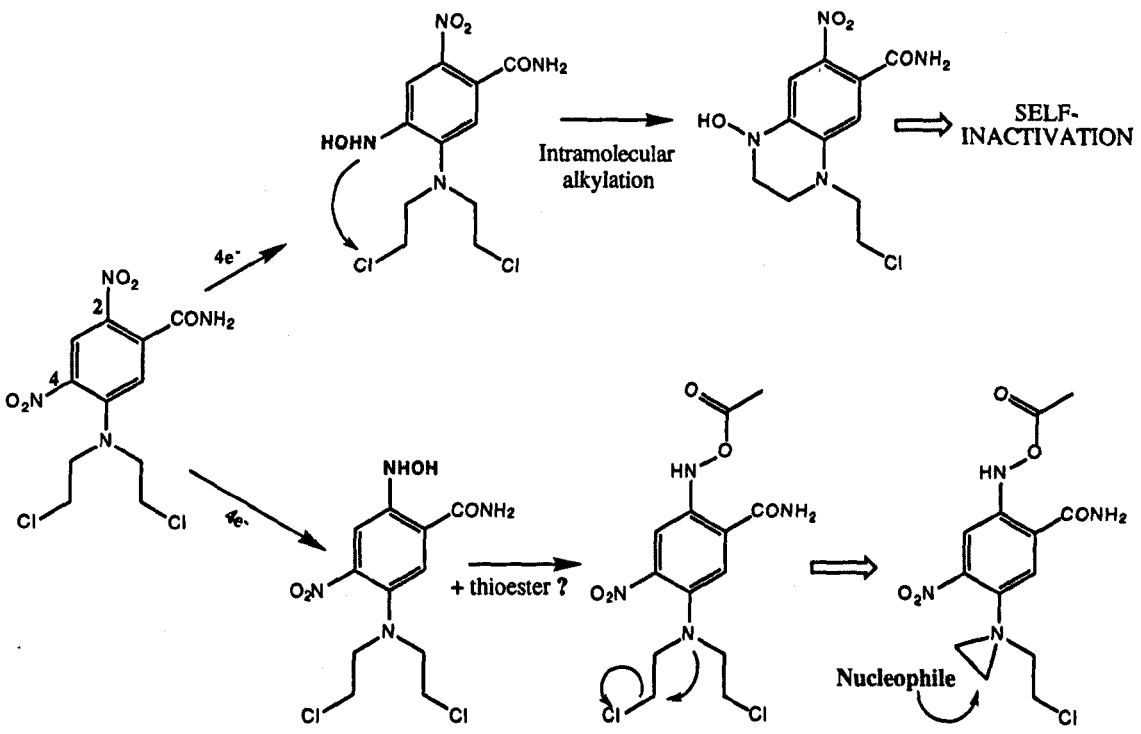
group of CB 1954, with the stoichiometry of 4- to 2-amine metabolite formation being ≈ 8:1 (Walton *et al.*, 1989). This probably reflects the fact that the 4-nitro group is the most electron-affinic site, and is consistent with evidence that radiolytic reduction of CB 1954 produces clean conversion to one major product, the 4-hydroxylamine (Palmer *et al.*, 1995). Experiments in hypoxic mammalian cells have also demonstrated that the major reductive metabolite is the 4-amine (Cliffe *et al.*, 1992; Simm *et al.*, 1997).

Figure 22: CB 1954 - differential 2- and 4- nitro reduction by human P450R and *E.coli* NR



Like CB 1954, the major SN 23862 metabolite formed by mammalian cells under hypoxic conditions appears to be the 4-hydroxylamine, although it is not known whether the reduction products of the 2-nitro group are recovered efficiently by HPLC (Cliffe *et al.*, 1992; Simm *et al.*, 1997). However, reduction of the 2-nitro group generates the dominant cytotoxic species since the 4-hydroxylamine spontaneously cyclizes via intramolecular alkylation to generate a stable ring-closed 6-nitroquinoxaline. This represents an inactivation mechanism and might explain the greater potency of the 2- vs. 4-amine chemical reduction products (Palmer *et al.* 1995).

Figure 23: SN 23862 - differential 2- and 4- nitro reduction



Radiolytic reduction of SN 23862 has indentified the 4-nitro group as the most electron affinic site (Palmer *et al.* 1995), suggesting, as with CB 1954, that P450R may preferentially reduce SN 23862 to the 4-hydroxylamine. However, the cytotoxicity of a 2-nitro analogue with a non-reducible (but equally electron-withdrawing) 4-methylsulfonyl substituent was found to be enhanced under hypoxic conditions, suggesting that endogenous oxygen-sensitive reductases can also reduce the 2-nitro group of SN 23862 (Atwell *et al.*, 1996). The comparable shift in sensitivity of the Rd-42 cell line to SN 23862 and CB 1954 under hypoxia (9.8- and 10-fold respectively), may be consistent with this proposal, and suggests that the putative 4-nitro reduction of SN 23862 by P450R is not a significant limitation. In agreement, the 4-amine metabolite was found to have a $t_{½}$ = 60 min. in culture media (pH 7), implying that the loss of compound via internal cyclisation will not significantly compromise cytotoxicity. Further, since efficient cell killing within a diffusion range of 200μM only requires a $t_{½} \approx 1$ min, 4-nitro activation will still allow extensive tissue diffusion (Denny and Wilson, 1993).

Both CB 1954 and SN 23862 are good substrates for *E.coli* NR and are lead candidate prodrugs for both NR-based gene and antibody directed prodrug activation therapies (Knox *et al.*, 1993; Anlezark *et al.*, 1995). Significant differences in metabolite profiles are observed with the NR activating system compared to that of P450R. *E.coli* NR reduces either nitro group of CB 1954 to its corresponding hydroxylamine with equal efficiency, such that only 50% of metabolism is therapeutically relevant. In contrast the only detectable NR-metabolite of SN 23862 is the cytotoxic 2-hydroxylamine product (Anlezark *et al.*, 1995; Friedlos *et al.*, 1997). Thus SN 23862 is currently being developed as a superior candidate *E.coli* NR-based GDEPT prodrug, although its clinical application is hampered by poor aqueous solubility. The addition of various hydrophilic side chains to SN 23862 has provided improved aqueous solubility (Palmer *et al.*, 1994), but analogues either retained hypoxic selectivity yet were no longer substrates for *E.coli* NR, or were highly activated by the enzyme but became poor hypoxic cytotoxins (Anlezark *et al.*, 1995).

6.4.3 Comparative efficiency of *E.Coli* Nitroreductase and human P450 reductase as nitro-prodrug activators.

Considering the contrasting metabolic preferences between P450R and NR with respect to both CB 1954 and SN 23862, it is perhaps relevant to compare their experimental utility as nitro-prodrug activators. Three publications, to date, have employed IC_{50} ratios in NR-expressing and non-expressing cells to provide a composite measure of the degree of reductive metabolism due to the enzyme and the relative cytotoxicities of the prodrug and its activated form (Green *et al.*, 1997; Friedlos *et al.*, 1997; 1998). Although variations occur in the cell line usage, the type of strong viral promoter driving transcription of the NR cDNA (in all cases CMV early immediate gene promoter) and the duration of prodrug exposure (24 or 72hr), broad comparisons are possible if several loose assumptions are made. Firstly we must ignore intrinsic differences in sensitivity between the various cell lines, although the published ranges of NR IC_{50} ratios provide some measure of the variation. Additionally, the comparison must assume the strength of the CMV (NR) and MLV LTR (P450R) promoters are roughly equal and both therapeutic proteins are expressed at equimolar levels. Since the background NR activity is nil in the parental cell lines, this is comparable with the IC_{50} values for parental MDA 231 cells following a 24h prodrug exposure under aerobic conditions (i.e. P450R-dependent activation is negligible), while the Rd-42 dose-response experiments were performed under 24h hypoxia. The absolute IC_{50} ratios for oxic parental vs. hypoxic Rd-42

were 221 and 39-fold for 24h CB 1954 and SN 23862 exposures, respectively. This corresponds to IC_{50} values of 288 μM vs. 1.3 μM for CB 1954, and 446 μM vs. 11.6 μM for SN 23862 (results not shown). Freidlos *et al.* (1997) found IC_{50} ratios for 24h exposure in V79 cells of 177 and 63-fold for CB 1954 and SN 23862, respectively. Similar IC_{50} comparisons in WiDr and SKOV-3 (human colon carcinoma and ovarian carcinoma cell lines) gave values of 52 and 225-fold for CB 1954 (Frieldos *et al.*, 1998). In a series of 72h exposure experiments, Green *et al.* (1997) recorded IC_{50} ratios for 72 h exposures in NR-positive and negative paired cell lines ranging from 17 to 517-fold (4 cell lines). No measure of catalytic activity was performed on these NR-expressing cell lines and the K_{cat} for these substrates has not been determined for P450R, so a more direct comparison is not possible. Nevertheless, this simplified approximation of the relative modulations in cytotoxicity conferred by therapeutic gene transfection and stable intracellular expression serves to illustrate the similar utility of the two enzymes as nitro-prodrug activators. The TE_{50} values calculated for P450R under hypoxia were also broadly similar to those seen for aerobic NR-dependent activation (Freidlos *et al.*, 1997; 1998), further illustrating the comparisons between the two prodrug-activation strategies.

6.4.4 Indole quinones as prodrug triggers.

All the indolequinone analogues were oxygen-inhibitable substrates for P450R, indicating they are efficient single-electron acceptors from P450R. The hypoxia-dependent sensitization in the Rd-42 cell line was variable among the five analogues; ranging from 5.7-fold for EO8 to 19.7-fold for analogue 2. However, elevations in P450R activity (eg. Wt *vs.* Rd-42) also influenced the aerobic cytotoxicty of the indolequinone-prodrugs, although this was less pronounced than seen under hypoxia, and varied from 1.3-fold for EO8 to 6.2-fold for analogue 3. Importantly, the modifications that remove any one of the three functional groups will reduce the number of reactive sites on the molecule, but may also change the reactivity towards DNA, and the final profile of cytotoxic lesions. However, it must also be considered that these differences may, in part, reflect changes in the rate at which the radical species of these indolequinone analogues disproportionate in relation to their oxygen sensitivity.

Under hypoxia, after one or two electron reduction, the hydroquinone and semiquinone forms of EO9 exist in equilibrium, with the equilibrium greatly favouring the hydroquinone ($K_{eq} > 4000$, pH 7.4) (Butler *et al.*, 1996):

$$Q^{\bullet-} + Q^{\bullet-} + 2H^{+} \leftrightarrow QH_2 + Q \quad \text{where } K_{7.4}^{EO9} = 5.2 \pm 1.6 \times 10^{7}\ M^{-1}\ s^{-1}$$

The rate constant is independent of the radical concentration in the range 1.0 - 5.2 μM and quinone concentrations upto 2.0 mM. However, this equilibrium would not be anticipated to be of significance in cellular systems under hypoxic conditions. Although it is possible that two semiquinones could react together and form a hydroquinone and a regenerated parent quinone, it is unlikely to be important at the very low nanomolar concentrations of drug required to kill cells (Butler *et al.*, 1996). The semiquinone radical, $EO9^{\bullet-}$, decays in the absence of oxygen over a period of milliseconds, in a dose dependent manner. In the presence of oxygen, $EO9^{\bullet-}$ is unstable and is rapidly oxidised:

$$Q^{\bullet-} + O_2 \rightarrow Q + O_2^{\bullet-} \qquad \text{where } K_{7.4}^{EO9} = 1.3 \pm 0.15 \times 10^{8}\ M^{-1}\ s^{-1}$$

These two rate constants are relatively similar, suggesting that spontaneous QH_2 formation by radical disproportionation might compete with backoxidation, thus rendering the one-electron reduction of the parent quinone potentially oxygen-insensitive. However, the vast molar excess of molecular oxygen over $Q^{\bullet-}$ in aerobic tissues ensures that backoxidation effectively out competes the possibility of radical disproportionation.

6.4.5 Comparative electrochemistry of the C-2 and C-3 positions or the indolequinone nucleus

A significant result was the fact that removing of the C-2 group (analogue 2) to generate a bi-functional agent, enhanced the hypoxia-dependent relationship between P450R activation and cytotoxicity, when compared to the tri-functional parent compound EO9 (19.7 and 9.4-fold respectively). This was reflected in the improved HCR values for analogue 2, which were amplified with increasing P450R expression. Taken together with the observation that analogue 2 was more potent than analogue 3 under hypoxia, the data suggests that the contribution of the hydroxymethyl group at C-3 was greater than the leaving group at C-2 under these conditions. Since both analogues are bi-functional, this difference may reflect the

ease by which a Michael-type elimination can initiate from the activated nitrogen in analogue 2 (figure 24), rather than the more dramatic movement of electrons from the semiquinone radical intermediate that is necessary to evict the leaving group (i.e. water) from the of hydroxypropenyl group of analogue 3 (figure 25).

DNA will behave as a nucleophile and attack the methide electrophile on the C-3 arm. Consequently the radical will alkylate DNA (Moody *et al.*, 1994.) If an aziridinyl group is present in the C-5 position it will rapidly react with the DNA to generate a DNA-DNA crosslink, believed to be the dominant cytotoxic lesion (Bailey *et al.*, 1994; Maliepaard *et al.*, 1995).

Figure 24: Proposed elimination of water from the C-3 hydroxymethyl group following the one-electron activation of analogue 2.

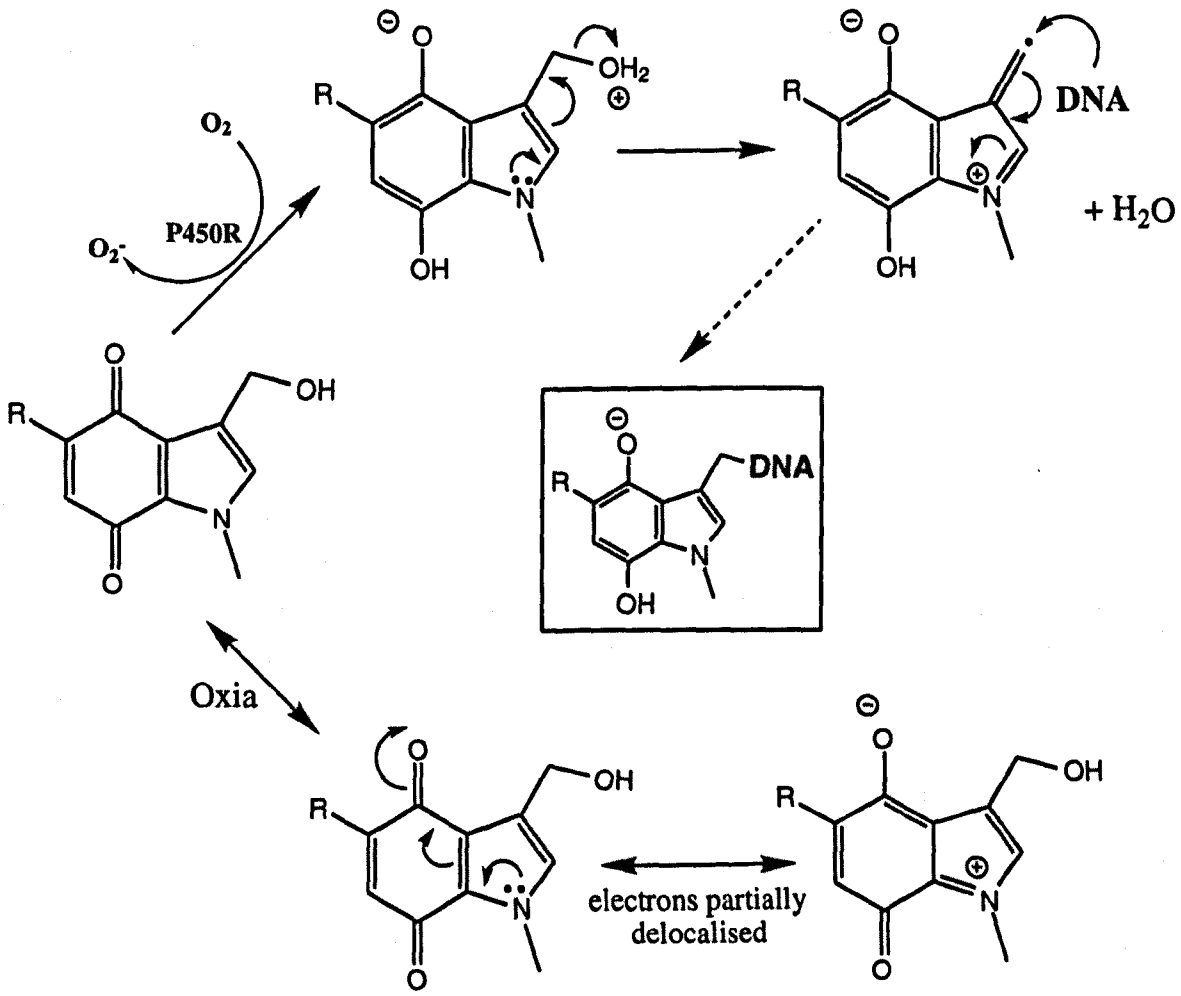
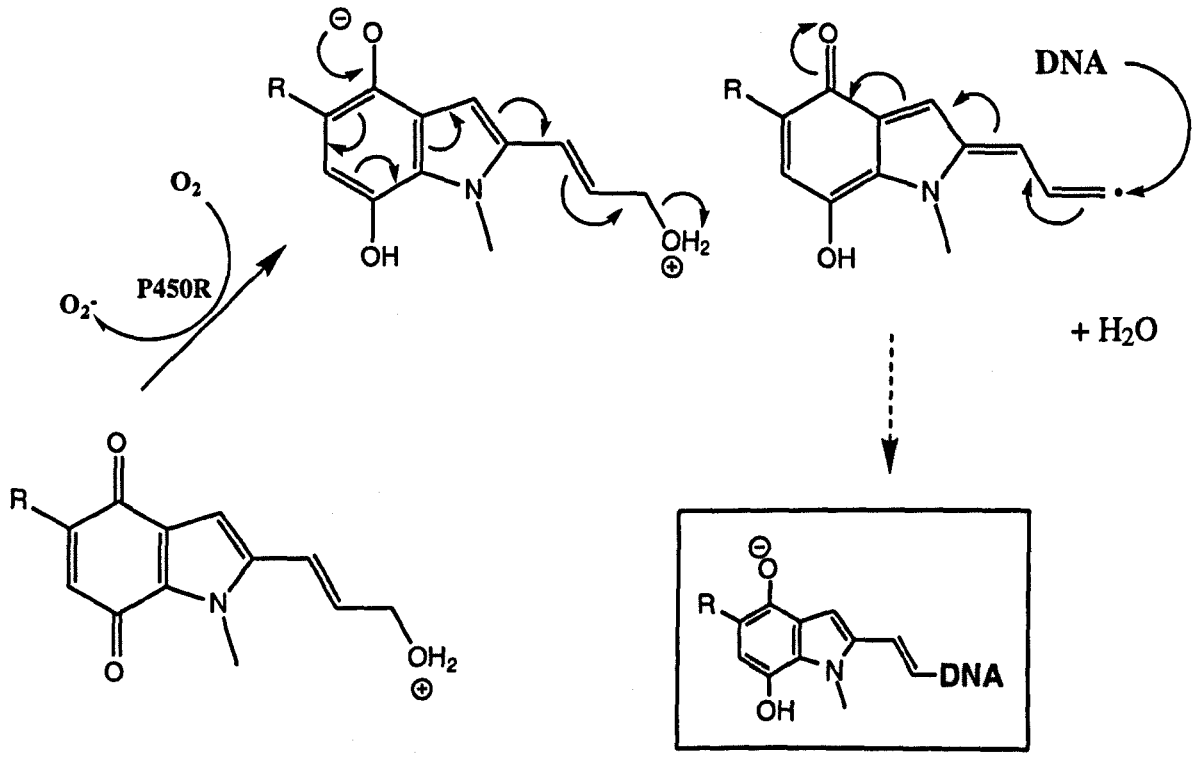


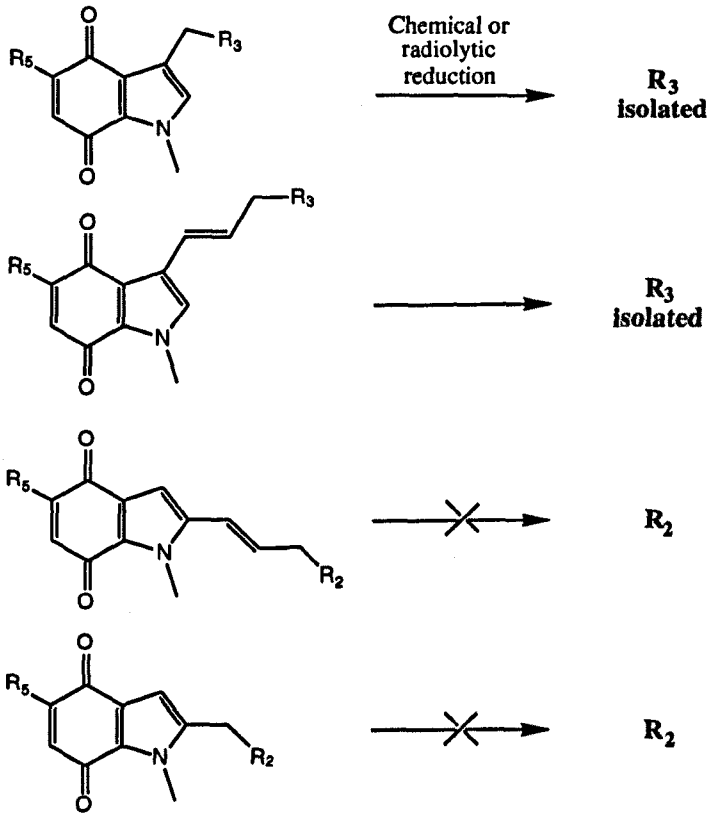
Figure 25: One-electron activation of analogue 3: Initiation of the elimination process from the semiquinone radical in order to evict water from the hydroxypropenyl C-2 group.



Interestingly, pulse radiolysis studies have suggested that the loss of the leaving group from a bioreductive quinone occurs only upon two-electron reduction to the hydroquinone and not at the level of the semiquinone radical (Wilson *et al.*, 1986). Yet a dichotomy is apparent between the chemical literature, where conditions favouring two-electron reduction are studied and alkylation mechanisms involving the hydroquinone have generally been proposed, and the biological literature, where most enzymes responsible for quinoid reduction transfer only a single-electron and mechanisms involving semiquinone formation leading to alkylation have been proposed. Since a quinone activated by an initial single-electron reduction will be in rapid equilibrium with its hydroquinone form, the difference may be more apparent than real. Recent evidence has suggested that the hydroquinone form of EO9 is unstable in air ($t_{½}$ =1.5 sec), resulting in the formation of the parent compound and H_2O_2 (Butler *et al.*, 1996):

$QH_2 + O_2 \rightarrow Q + H_2O_2$ where $K^{EO9}_{7.4} = 2.1 \pm 0.4 \times 10^3\ M^{-1}\ s^{-1}$

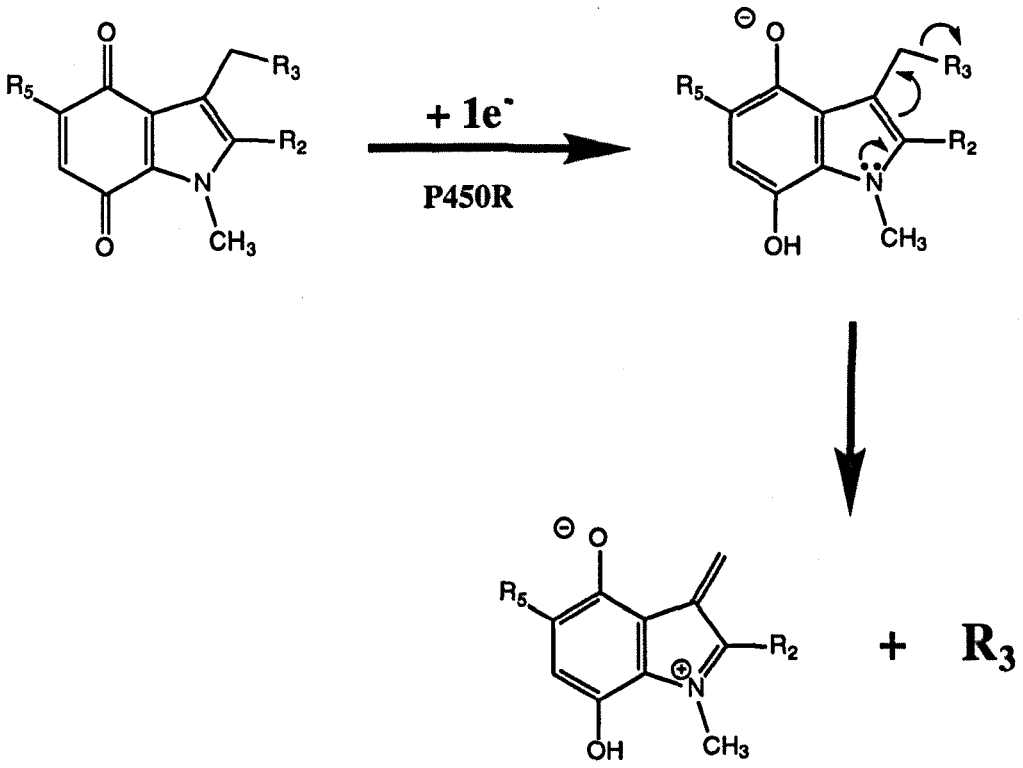
Radiolytic reduction studies of a number of 5-methoxy analogues have confirmed that a range of leaving groups located in the C-3 position undergo efficient Michael-like elimination, while the C-2 position fails to function as a site for elimination (Jaffar *et al.*, unpublished observations). Furthermore, the efficiency of elimination from the C-3 position can be related to the pKa value of the leaving group.



These results taken together with the *in vitro* cytotoxicity data, suggest a model by which a diffusible cytotoxin can be linked to the indolequinone nucleus, so as to remain latent until the compound is reduced under anoxia to the semiquinone radical form. Utilisation of a cytotoxic leaving group with both a desirable pKa value, and an inherent latency as a consequence of electron withdrawal (eg. an aliphatic nitrogen mustard), will ensure that futile cycling of the indolequinone in the presence of oxygen does not result in the inappropriate

release of the "effector". The very rapid kinetics of backoxidation of semiquinones ensures the hypoxia specificity of this process (Wardman *et al.*, 1995; Bulter *et al.*, 1996).

Figure 26: Proposed model for exploiting the indolequinone nucleus as an oxygen-inhibited "trigger" to release an oxygen-insensitive diffusible cytotoxin.



6.4.6 Avoiding aerobic bioactivation

An important aspect of this model is to develop indolequinone prodrugs that will not be substrates for the two-electron reductase, DT-diaphorase. Critically, metabolism by this enzyme will diminish the specificity for hypoxia cytotoxicity, and as discussed earlier, may actually confer protection from EO9 cytotoxicity under hypoxia (Plumb *et al.*, 1994b). The observations of increased expression of DTD following acute hypoxia (Yao *et al.*, 1994) suggest a potential conflict could arise in hypoxic tissues if the hypoxia-targeted prodrug is significantly metabolised by DTD. Moreover, the high basal levels of DTD activity found in

normal human tissues such as the kidney, stomach, breast and colon (Schlager and Powis, 1990; Riley and Workman, 1992; Belinsky and Jaiswal, 1993; Smitskamp-Wilms *et al.*, 1995, 1996), clearly constitutes a major problem when attempting to selectively target bioreductive drug metabolism to tumour tissues. The intrinsically high DTD levels in the human kidney may account for the nephrotoxicity observed in Phase I trials of EO9, with proteinuria being the dose-limiting toxicity (Schellens *et al.*, 1994). Similarly, the very high DTD activity in stomach and colon tissues of the mouse (Schlager and Powis, 1990) might be related to the dose-limiting gastrointestinal toxicity observed with EO9 in murine studies (Hendriks *et al.*, 1993). Additionally, DTD (and other phase I and/or phase II enzymes) is strongly induced (upto 300-fold) by a wide variety of chemical agents and dietary constituents including dioxin, polyaromatic hydrocarbons, azo dyes, flavonoids, diphenols, isothiocyanates, dithiolthiones and thiocarbamates (Prochaska *et al.*, 1985: Prochaska and Talalay, 1988). It is thought that the induction of DTD is regulated, in part, by promoter elements including the antioxidant response element (ARE) (Li and Jaiswal, 1992a; Belinsky and Jaiswal, 1993).

The problem of tissue selectivity is perhaps illustrated by the clinical history of the aminoquinone antibiotic, streptonigrin, which is an exellent substrate for human DT-diaphorase (Beall *et al.*, 1994; Ross *et al.*, 1996) and was shown to have the best correlation between DT-diaphorase activity and cytotoxicity of over 31,000 compounds tested in the NCI human tumour cell line panel (Fitzsimmons *et al.*, 1996). Streptonigrin given either intravenously or orally was evaluated against a wide range of human tumours and at the maximum tolerated dose produced objective responses only in patients with lymphoid malignancies (Review: Kremer and Laszlo, 1975). However the high degree of toxicity, in the form of severe nausea and vomiting and life-threatening delayed bone marrow suppression, led to its abandonment for clinical trial (Humphrey and Blank, 1961).

The design of indoloquinone prodrugs that are not metabolised by DT-diaphorase has already been partially achieved through modification of the aziridine at the C-5 position (R_5). Both EO8 and EO7 are poor substrates for DT-diaphorase, and are reduced 7.6 and 13.1-fold less efficiently than EO9, respectively (table 7) (Jaffar *et al.*, 1998a). These changes in DTD substrate specificity (and aerobic cytotoxicity) might in part reflect changes in the two-electron reduction potentials of these compounds, but more likely suggest the introduction of marked steric hinderance effects at the active site of DTD.

Table 7: EO9 and four related analogues: Substrate specificity for human DT-diaphorase and one-electron reduction potentials at pH 8.5.

Indoloquinone analogue	Specific activity (μM / min / mg)	$E_{8.5}(1)$ (mV)
EO9	19.90 ± 0.50	- 265
Analogue 2	52.78 ± 0.93	- 277
Analogue 3	41.59 ± 1.08	- 276
EO8	1.52 ± 0.14	- 284
EO7	2.62 ± 0.93	- 309

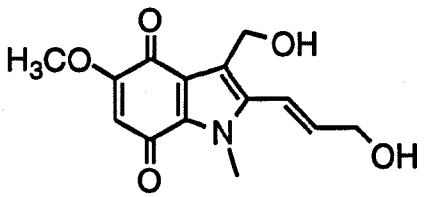
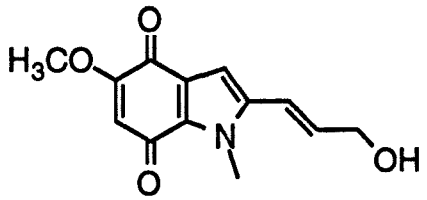
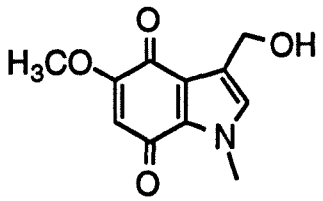
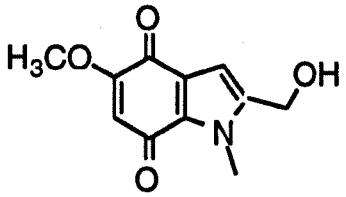
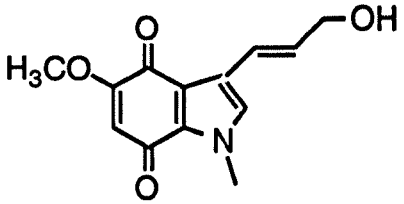
More recent work has also identified the C-2 position of the indoloquinone backbone (R_2) as an important determinant of DTD substrate specificity (data not shown), and several analogues have been identified that are either exellent or poor substrates for this enzyme (Specific activity DTD 127 - 0.3 μM/min/mg). In general terms, introduction of a bulky chemical moiety, such as a phenyl group can markedly increase DTD specificity. These modifications can also incorporate increased aqueous solubility into the structural design, and, importantly, can improve the pharmacokinetic stability of the parent molecule in murine studies (chapter 7).

6.4.7 Role of the C-5 position in determining potency and enzyme specificity

Since the C-5 aziridinyl is the major contributor to the potency of the indoloquinone analogues (Bailey *et al.*, 1992; Phillips 1996; Naylor *et al.*, 1997; Jaffar *et al.*, 1998a, 1998b), it may be desirable to remove this reactive group when designing a non-toxic trigger molecule for the release of a cytotoxic effector. In this respect it is fortuitous that the displacement of the aziridinyl for a methoxy group not only renders the compound a poor substrate for DT-diaphorase, but also favourably raises its one-electron reduction potential. In the case of the C-5 methoxy, EO7, potential alkylating functionality is still retained at both the C-2 and C-3 positions, and this is necessary to achieve any appreciable hypoxia-specific cytotoxicity. Since modification of the C-2 group (or replacement with a non-alkylating group) will prevent the molecule from crosslinking DNA, it effectively lowers the HCR of the

indolequinone nucleus (Jaffar *et al.*, 1998a). Thus the cytotoxicity arising as a consequence of futile cycling of the semiquinone / hydroquinone in the presence of oxygen becomes as significant as that resulting from monoalkylation by the indoloquinone itself.

Table 9: Sensitivity of V-79 cells to EO7 and its mono-substituted regioisomers in the C-2 and C-3 positions. (From: Jaffar *et al.*, 1998a).

EO7 Regioisomers	IC_{50} (μM) Oxic	Hypoxic	HCR
	851	30	27.8
	500	30	16.7
	220	240	0.9
	633	430	1.5
	360	260	1.4

The low potency of the 5-methoxy analogue of EO9 enables the linkage of an effector, such as a mustard, which is ≈ 500-fold more cytotoxic than the trigger. For example the acute (3h) aerobic IC_{50} value for EO7 in the V-79 cell line is 850 μM, while the IC_{50} for acute (3h) hypoxic IC_{50} is 30 μM (i.e. HCR= 27.8), (Jaffar *et al.*, 1998a). This compares to the IC_{50} value (3h) for a proposed phosphoramide dichloro-mustard leaving group of 1.7 μM, in this cell line. Alternatively, bromo- and iodo-mustard effectors could be utilised, since they are 50 to 75-fold more potent than their corresponding chloro-mustards and can transmit their toxicity to neighbouring cells with much greater efficiency *in vitro* (Friedlos *et al.*, 1997). It might be argued that if the trigger itself is highly potent but non-diffusible, it will preferentially kill the transfected cells that are overexpressing the reductive enzyme *in vivo*, compromising the production and distribution of the diffusible effector, and perhaps limiting the therapeutic window of prodrug administration. Modification of the C-5 position with other inactive moieties is currently being studied since it appears to be an important determinent of both DTD substrate-specificity and by implication, the aerobic (normal tissue) cytotoxicity.

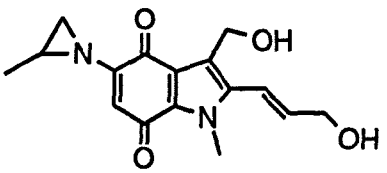
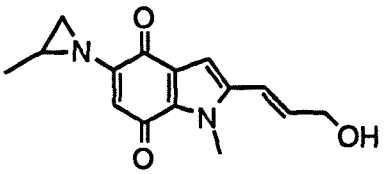
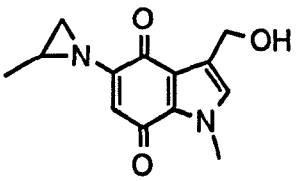
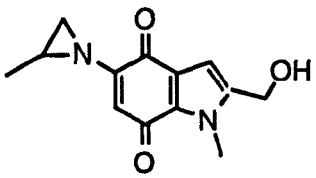
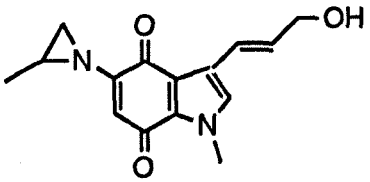
In agreement with these observations, the importance of the C-5 position for determining both DTD substrate specificity and aerobic cytotoxicity has recently been confirmed (Phillips, 1996) and is in broad agreement with earlier findings (Bailey *et al.*, 1992). Structure-activity studies (Phillips, 1996) demonstrated than relatively minor changes at C-5 can have a major effect on both DTD substrate specificity and aerobic potency. Of six EO9 analogues evaluated, only two retained specificity for human DTD. Of particular relevance, replacing the aziridine ring of EO9 with a phenyl-amino group ($-NHC_6H_5$) lead to the total inability of purified human DTD to metabolise the analogue and complete loss of aerobic cytotoxicity. In contrast, replacing the hydroxyl group in the C-2 position of EO9 with the phenyl-amino group had little effect on DTD substrate specificity and did not influence aerobic potency. The potential contribution of one-electron reductases to metabolism and the HCR values of these analogues were not reported.

6.4.8 Chemical and biological factors relevent to the design of appropiate quinone-triggered diffusible prodrugs

It may be advantagous to retain the potency of the bioreductive trigger in the design of a hypoxia-activated diffusible cytotoxin. The utility, for example, of the methyl-aziridinyl group (EO8) in lowering the substrate specificity for DT-diaphorase while retaining specificity for activation by one-electron reduction might be, in principle, a desirable property. An analogue of EO8 supporting a latent diffusible cytotoxin in the C-3 position, would function both as a conventional quinone methide precursor and as a hypoxia-activated trigger for a diffusible cytotoxic effector. This would allow the trigger-effector combination to target both the hypoxic and aerobic compartments of a tumour mass, and might have significant utility when combined with ionising irradiation. Thus a bioreductive trigger can have a dual role; the potent trigger ensures that the radio-resistant acute hypoxic subpopulation of a solid tumour is targeted, while its oxygen-sensitive electrochemistry colaterally exploits the unique physiology of the tumour to release a diffusible cytotoxin. Lethal exposure of the hypoxic cells to the trigger unit, while eventually manifesting itself as a loss of clonogenicity, should not compromise the short term metabolic functions of cells. The ability to maintain metabolic integrity and successfully divide several times following lethal free radical damage is a well documented property of neoplastic cells (Hall, 1994).

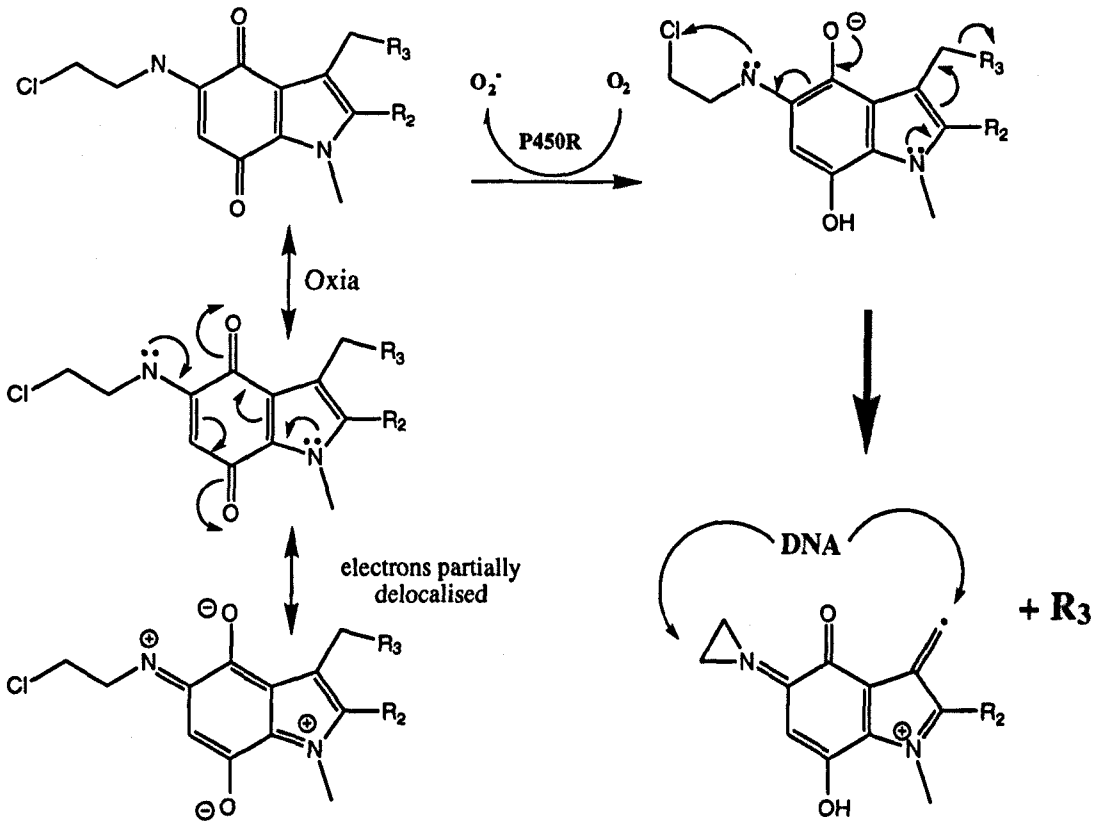
The key requirements of a novel co-therapeutic trigger-effector design is that the effector unit must be significantly more potent than that of the trigger under aerobic conditions, and ideally the trigger should have a large HCR value. This is necessary to allow the administration of therapeutically relevant doses of the compound, with respect to the released effector function. In the case of EO8, this is made possible by adapting the linker arm that inactivates the effector, in order to limit the aerobic potency of the quinone but retain toxicity following eviction of the leaving group. This is readily achieved by manipulating the energetics of electron redistribution through the linker, coupled with an appropriate electron-affinity for the effector i.e. its willingness to leave (pKa). The design principle of the linker is exemplified in the loss of aerobic potency for both of the hydroxypropenyl substituted EO8 regioisomers in the rodent V-79A cell line (Jaffar *et al.*, 1998a). Thus it should, in principle, be possible to design novel analogues of EO8 that retain the impressive HCR, while reducing the aerobic potency of the molecule.

Table 9: Sensitivity of V-79 cells to EO8 and its mono-substituted regioisomers in the C-2 and C-3 positions. (From: Jaffar *et al.*, 1998a).

EO8 Regioisomers	IC_{50} (μM) Oxic	Hypoxic	HCR
	57.7	0.011	5245
	153	5.6	27.2
	4.4	0.18	24.4
	54	1.8	30.0
	276	13.1	21.1

The C-5 methyl-aziridine, although considerably less active than the aziridine functionality of EO9 under aerobic conditions, still dominates in determining the potency of EO8 under both aerobic and hypoxic conditions. Therefore other modifications have been considered to improve the aerobic latency of the 5-position, including utilising alternative chemistry to provide inactivation of the aziridinyl group. One example is the use of a mono-functional nitrogen mustard. These aspects of trigger design are currently being studied, and a schematic representation of the one-electron activation pathway is shown in figure 27.

Figure 27: Schematic representation of an indoloquinone based, dual-function hypoxic cytotoxin and hypoxia-activated diffusible cytotoxin.

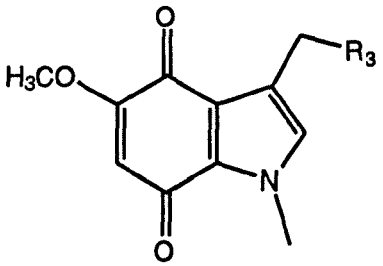


In this example R_3 could be a phosphoramide or aliphatic nitrogen mustard, while R_2 can be a hydrophilic side chain which does not function as a leaving group, but importantly, contributes to both substrate specificity and pharmacokinetic stability. One significant limitation in the rational design of indolequinone based hypoxic cytotoxins with additional diffusible leaving groups, is the lack of predictability regarding the structural requirements essential in the generation of exclusive one-electron reductase substrates. This has been exemplified for a series of EO9 analogues, where relatively minor structural changes have resulted in major changes in substrate specificity for human DT-diaphorase, which were strongly interrelated with similarly dramatic modifications of aerobic cytotoxic potency (Phillips, 1996). Consistent with the lack of predictability, no correlation was found between reduction potential and rates of reduction by DT-diaphorase for a series of napthoquinones (Buffington *et al.*, 1989) or a series of aziridinylbenzoquinones (Gibson *et al.*, 1992).

Resolution of the X-ray crystallographic structure of human DT-diaphorase will aid in the design of indoloquinone analogues that do not conform with the catalytic requirements of this enzyme's active site. So far the crystal structure of rat liver DT-diaphorase has been determined to 2.1Å resolution and is published in preliminary form (Li *et al.*, 1995).

Despite the lack of predictability, early DTD structure-activity studies have provided insight into the importance of the C-3 position (leaving group) chemistry. Any increases in bulkiness of R_3 renders the indolequinone a poorer substrate for human DTD. This is a desirable property in view of the large effector groups that would be desirable in this position.

Table 10: Influence of R_3 on DT-diaphorase specificity of 5-methoxy-[1*H*-indole-4,7-dione].



R_3	Human DTD specificity (μM/min/mg)
OH	25.0
Cl	0.17
OCOAr	0.26
OCOAr'	0.29
OCOAr"	0.66
OAr	0.50

Ultimately, the application of this knowledge in rational prodrug design, when coupled with the minimal design constraints necessary to for these agents to be oxygen-sensitive electron acceptors, will guide the enzyme-directed prodrug synthesis programme and may yet prove fruitful.

Chapter 7

7. Discussion.

7.1 The potential clinical role of gene therapy in local disease control.

The need for local control in the cure of cancer remains a matter of crucial importance. At diagnosis approximately 70% of patients have no detectable distant metastasis and over half are cured by loco-regional treatment only (Brady *et al.*, 1990). Since $^1/_3$ of patients die as a consequence of failure to control loco-regional disease (Suit, 1982), therapeutic strategies aimed at this patient sub-population are a source of potential progress.

Importantly, securing local control is related to the reduction in the incidence of metastatic spread, since local residual disease may constitute a nidus for distant dissemination. This has been observed for several types of cancer including; breast (Tubiana *et al.*, 1986), cervix (Anderson and Dishe, 1980), prostate (Fuks *et al.*, 1991) and head and neck (Leibel *et al.*, 1991). For example, in breast cancer the probability of distant dissemination increases from 15% to 75% when the diameter of the tumour increases from 1.5 cm to 5.5 cm (Koscielny *et al.*, 1985). Thus clinical evidence suggests that local recurrence is a step on the path to metastatic spread, and therapeutic progress will come from the introduction of more effective local control.

In western Europe and the USA (DeVita, 1983), of the patient sub-population that present with localised disease, approximately 22% are cured by surgery, 18% by radiotherapy, but only 5% by chemotherapy. This is despite the development and clinical exploitation of a wealth of novel chemotherapeutic agents in the last 20 years. These disappointing figures, result in part, from the fact that surgery and radiotherapy are localised regimens, whereas chemotherapy is a systemic treatment and its toxicity, particularly against bone marrow, limits the tolerated doses of drug that can be administered. This inevitably leads to sub-optimal concentrations, particulary in tumour cell sub-populations with acquired or intrinsic resistance.

Such resistance is multi-factorial (chapter 1), and a recognised component of these mechanisms is the presence of areas of low oxygen tension within the solid tumour mass (Gatenby *et al.*, 1988; Hockel *et al.*, 1991; Vaupel, 1991). Tissues within this micro-enviroment can frequently remain viable, contributing significantly to both radio- and chemo-

resistance (Gatenby *et al.*, 1988; Hockel *et al.*, 1993; Okunieff *et al.*, 1993; Teicher, 1994). As a result, cells within this aberrant enviroment may ultimately become active clonogens, which repopulate the tumour and facilitate the relapse of a multidrug-resistant cancer phenotype (Review: Kallinowski, 1996).

Therefore, therapeutic strategies aimed at delivering acute localised concentrations of cytotoxic agents to these clinically resistant solid tumour sub-populations, without concurrent systemic complications, could represent a fundamental clinical gain, improving not only the efficacy of chemotherapy treatments but overall patient quality of life. "Targeted" chemotherapy is possible through an understanding of the role of potential therapeutic enzymes and the inert prodrugs that they can metabolise (Senter *et al.*, 1993). If such knowledge is complemented by tissue-specific expression stategies, to localise production of a therapeutic protein only within a defined tissue enviroment, such selectivity can be achieved. In addition, the successful application of molecular chemotherapy in cancer will require high level gene expression in a tumour-specific manner, ideally combined with highly selective therapeutic gene delivery (Anderson, 1984; Spooner *et al.*, 1995; Anderson, 1998). The efficient delivery of therapeutic DNA is becoming a realistic possibility through advances in the efficiency of gene delivery to tissues, utilising techniques such as "gene-gun" inoculation (Cheng *et al.*, 1993), lipofection (Felgner *et al.*, 1987), virus-mediated delivery systems (Vile and Russell, 1994), receptor-mediated endocytosis (Cristiano *et al.*, 1993) and replication-incompetent adenovirus co-internalisation (Cotten *et al.*, 1992). Some of these delivery strategies have resulted in favourable increases in the amplitude of tumour-specific gene delivery and subsequent expression, a factor that has clear implications for any therapeutic enzyme based prodrug treatment.

7.2 The potential to target metastatic disease.

Several lines of evidence suggests that micrometastasis can potentially contain regions of hypoxia that can stimulate neovascularisation and initiate the capillary networks necessary to provide an adequate nutrient supply (Craft and Harris, 1994; Weinsat-Saslow and Steeg, 1994; Folkman, 1995). However a major limitation in the application of oxygen-sensitive GDEPT to disseminated disease is the ability to efficiently deliver therapeutic DNA. Yet in some cases it may prove possible to deliver therapeutic genes through cell surface receptors, such as the epidermal growth factor receptor (EGFR), which can be upregulated by low tissue pO_2 (Laderoute *et al.*, 1992a; 1992b). DNA delivery via EGFR-mediated endocytosis

(Cristiano and Roth, 1996; Michael and Curiel, 1994), or through ligand-directed retroviral targeting (Chu *et al.*, 1994; Han *et al.*, 1995; Cosset and Russell, 1996) might then target metastatic disease that is susceptible to periods of transient hypoxia. Alternatively, anaerobic spore-forming bacteria such as the *Clostridium* genus could be employed to provide hypoxia-specific therapeutic gene delivery (Minton *et al.*, 1995; Fox *et al.*, 1996b).

These hypoxia-responsive gene delivery approaches could be complemented with the utilisation of alternative splicing, such as observed for CD44 isoforms (Reviews: East and Hart, 1993; Zöller, 1995). The CD44 variant isoforms (CD44v) can promote the metastatic behaviour of cancer cells, and strategies that incorporate the alternative splice control elements into therapeutic gene constructs can target preferential pre-mRNA processing of therapeutic gene/CD44 chimeras to metastatic cells (Asman *et al.*, 1995; Cooper *et al.*, 1996).

7.3 Cytochrome P450 reductase as an oxygen-sensitive prodrug activator.

Among the potential oxygen-sensitive mammalian reductases, P450R is the most clearly characterised with respect to the biochemical specificity of one-electron transfer, and this is reflected in the wealth of publications identifying its role in the hypoxia-specific activation of a wide range of bioreductive prodrug substrates. The unique chemistry conferred by the presence of two cooperative flavin prosthetic groups (Vermilion and Coon, 1978a), provides a distinction between P450R and other flavoenzymes such as aldehyde oxidase, XO, XDH and B_5R. These latter reductases contain only a single FAD unit as the proximal redox centre (Rajagopalan and Handler, 1967; Iyanagi, 1977), and appear to utilise this prosthetic group as both the electron inlet and outlet site when reducing artificial electron acceptors (prodrugs). Since the reaction of NADH with FAD is an obligatory two-electron reduction, $FADH_2$ is, by implication, the immediate product (Kanda *et al.*, 1972; Powell and Bruice, 1983a, 1983b). The subsequent outcome of a one- vs. a two-electron reduction of a bioreductive prodrug will then depend upon the redox enviroment of the flavin within its polypeptide microenviroment, the resulting equilibrium between the flavin semiquinone and hydroquinone forms and their stability with respect to autooxidation. The implication must also be that cofactor and drug substrate will be in direct competition for the same redox site in the enzyme. In contrast, P450R accepts two-electrons from NADPH at the FAD prosthetic group, whereas FMN transfers electrons to the acceptor, through the stable $FMNH^{\bullet}$ semiquinone form (Vermillion and Coon, 1978b; Yasukochi *et al.*, 1979). The necessity for intramolecular electron transfer in order to facilitate single-electron availability is suggested by the fact that

FMN-depleted P450R, while readily reducible by NADPH, is unable to transfer an electron to quinone acceptors (Vermillion and Coon, 1978b). This is in agreement with kinetic studies on the individually encoded and expressed domains of P450R, where the truncated FAD-containing domain of P450R is rapidly reduced by NADPH, but can not in turn reduce model quinone substrates such as menadione (Smith *et al.*, 1994). The unique redox chemistry of P450R, coupled with an inherent flexibility in prodrug metabolism provided by its capacity to donate an electron to any artificial acceptor with an appropriate reduction potential, makes P450R a promising candidate for oxygen-sensitive GDEPT.

The potential for therapeutic gain following the introduction of a P450R-expressing therapeutic construct will depend in part upon the background levels of endogenous P450R, as well as the presence of tissue hypoxia. Tumour hypoxia, and the ability to manipulate it, has been discussed in chapter 1. The contribution of other reductive enzymes, such as CYP450 and DTD, may also play a role in determining tissue specificity, but this may depend upon the choice of prodrug substrate. However, currently only limited information regarding reductive enzyme expression in human solid tumours is published.

P450R appears to be widely distributed in human tissues. This has been documented immunohistochemically in the liver acinus, enterocytes of the small intestine and colonic epithelial cells, pancreatic ductal cells and a number of cellular types in the lung and kidneys. This distribution was similar to that found in laboratory animals but the reductase localisation in the kidney was more extensive in humans than that seen in the rat, guineapig and rabbit (Hall *et al.*, 1989). In the presence of sufficient oxygen, the broad normal tissue expression of P450R should be of little therapeutic relevence with respect to the specificity of targeted prodrug activation.

Rampling and colleagues (1994) conducted reductive enzyme profiling for P450R, b_5R and DTD in human malignant gliomas, revealing a broad spectrum of activities, with values ranging by 8, 11 and 18 fold respectively. A similar heterogeneity in functional P450R activity was documented in the primary breast biopsies. Immunohistochemically, the staining for P450R appeared to be greater in the peritumoural normal tissues, suggesting down-regulation in some samples (chapter 3). However P450R activities of paired normal tissues was not conducted. Interestingly, the reduction in P450R expression has also been documented in a chemically-induced lung tumour model, with reductions in expression being associated with advancing stage and progression of the lung neoplasia (Forkert *et al.*, 1996). Reduced expression of P450 reductase relative to the adjacent normal tissues has been reported in

human primary and secondary liver tumours (Philip *et al.*, 1994). Thus, the available data would suggest that significant scope may exist for the re-introduction of high P450R activity into hypoxic tumours for therapeutic gain.

The CYP450 system exhibits a more limited and tissue-specific expression pattern. A distinctive pattern of CYP450 down-regulation has been reported in human primary and secondary liver tumours, showing reduced expression of CYP450 isozymes 1A1, 2A6, 2B6, 2C8/9, 3A4, and 4A, relative to the adjacent normal tissue (Philip *et al.*, 1994). Similarly, de Waziers *et al.* (1991) found significant decreases in CYP450 3A4 in human colonic tumours compared to adjacent normal tissues, with CYP450 1A1, 1A2, 2C8/10 and 2E1 being undetectable by western blotting in either tumour or normal tissue pairs. Further evidence for the low level of CYP450 in human tumours has been found by Forrester *et al.* (1990). They compared the expression of CYP450 in 41 breast cancer samples relative to the surrounding normal tissues. Only CYP450 2C was detected by Western blot analysis, with no CYP450 1A2, 2A6, 2B6, 2D6 and 3A immunoreactivity being apparent. In contrast to western blotting results, staining of tumour sections revealed significant CYP450 3A immunoreactivity.

In some cases, CYP450 has been shown to be expressed in human tumours without any expression in the adjacent normal tissues. Murray *et al.* (1993) immunohistochemically detected CYP450 in a series of tumour samples without revealing any staining of the matched normal tissue samples. In breast cancer, 39% were found to be positive for CYP450 1A and 22% for CYP450 3A, whereas 70 and 78% were found to stain positively for CYP450 1A and 3A respectively in soft tissue sarcomas (Murray *et al.*, 1993a; 1993b). Recently this group also showed neoplasia-specific staining for CYP450 1B1 in a wide range of human tumour samples, again without any detectable immunoreactivity in adjacent normal tissues (Murray *et al.*, 1997).

In general DTD activity is higher in tumours than the surrounding normal tissues (Ross *et al.*, 1993; 1996). Schlager and Powis (1990) showed that elevated levels of this enzyme have be found in primary colonic, breast and lung carcinoma as well as human hepatoma, whilst renal cell and gastric adenocarcinomas had low DTD activities compared to adjacent normal tissues (Schlager and Powis, 1990). De Waziers *et al* (1991) showed that DT-diaphorase levels were unchanged in colorectal adenocarcinoma compared to the surrounding normal colonic tissue. However cumulative *in vitro* evidence would suggest that differing levels of tumour DTD expression will have little influence upon the efficacy of TPZ treatment.

Thus, with the notable exception of DTD activity, reductive enzyme profiles are often down-regulated in the neoplastic state, suggesting that the re-introduction of high levels of expression of specific single-electron reductases, such as P450R, preferably in a compementary oxygen-sensitive gene expression strategy, could provide a valid approach to targeting bioreductive prodrug activation to hypoxic tumour tissues.

7.4 Choice of P450 reductase prodrugs

The nitro-prodrugs are now well advanced as GDEPT substrates in combination with *E.coli* NR. Their well defined mode of activation, their proven mechanism of cytotoxicity as well as their high specificity for NR-mediated catalysis, will probably ensure rapid development towards clinical trials. In particular, the limited metabolism by normoxic tissues (particularly the mustard derivatives) should contribute towards the selectivity of these agents in both GDEPT and ADEPT strategies. If the efficacy of P450R-dependent GDEPT in combination with these prodrugs can also be demonstrated *in vivo*, particularly in combination with anti-vascular strategies, this could extend the potential role of these hypoxia-activated diffusible cytotoxins in GDEPT of solid tumours.

While the principle of exploiting indolequinones as hypoxia-activated trigger units has been demonstrated, much work is required to define the prodrug characteristics that are suited to an oxygen-sensitive GDEPT strategy. In particular, a very low or absent DTD substrate specificity is essential, and the pharmacokinetic properties of any such prodrug analogues must be a primary consideration. Nevertheless, the theoretical advantages that a indolequinone-triggered prodrug might offer, must make the search for such analogues a valid drug development strategy.

7.5 Role of quinone-triggered bioreductive prodrugs and their potential in oxygen-sensitive GDEPT

Delivering therapeutic genes to hypoxic cells within a solid tumour mass is a fundamental hurdle to any oxygen-sensitive GDEPT strategy. Systemic targeting of the diffusion-limited hypoxic tumour tissues is by its nature impeded by the distance of such tissues from the functional vasculature. It is thus technically more desirable to target the dynamically fluctuating "acute" hypoxic tumour cell population. As reviewed in chapter one, interruptions in blood flow generate transient foci of acute hypoxia in tumour tissues normally

proximal to well established microvascular networks (Reinhold *et al.*, 1977; Brown, 1979). Studies in human tumour xenograft models indicate that perfusion-limited hypoxia is a common phenomenon (Chaplin and Trotter, 1990), and clinical analysis of a variety of tumours of different histologies, have confirmed these experimental observations (Hill *et al.*, 1996). Critically, the observation that radiobiologically hypoxic cells can result from dynamic perfusion changes (Chaplin *et al.*, 1987) lends support to the inference that acute hypoxia can arise from changes in vascular flux, and targeting cells that are proximal to the vascular supply is a valid hypoxia-directed GDEPT strategy.

While experimental data suggest these transient periods of acute oxygen deprivation can give rise to severe radiobiological hypoxia (i.e. anoxia; < 10 ppm. O_2), the duration of this deprivation can be limited. Therefore upon establishment of this acute hypoxic microenvironment, it is clearly advantageous if an applied prodrug substrate is very rapidly metabolised. Consequently, the fact that quinone radicals disproportionate up to four orders of magnitude faster than nitro radicals has obvious theoretical advantages when considering the design of oxygen-sensitive prodrug triggers as delivery vehicles for diffusible cytotoxins. However the design constraints imposed by the use of the indolequinone nucleus are greater than that of the nitro-aromatic compounds, and suffer from the necessity to design out any DTD substrate-specificity as well as ensuring good pharmacokinetic properties *in vivo*. Nevertheless, the permutations of prodrug design are potentially broad enough to allow the rational development of sub-classes of quinone analogues that conform to these desired properties.

7.6 Pharmacokinetic considerations in rational indolequinone prodrug design

A major factor in the efficacy of chemotherapeutic agents is appropriate pharmacokinetic stability *in vivo*. This property is particularly relevant to indole quinones. Pharmacokinetic metabolism depends, for the most part, upon the nature of the functional groups surrounding the quinoid nucleus. This is exemplified by an analogue of the anthracycline doxorubicin (Adriamycin), 4'-epidoxorubicin (Epirubicin), where a small change in the stereochemistry of the daunosamine sugar results in a large difference in the levels of glucuronide conjugate formation. This lack of glucuronide formation by doxorubicin compared to 4'-epidoxorubicin arises through the axial C-4'-hydroxyl of doxorubicin being sterically hindered by an adjacent *cis* equatorial 3'-amino group, which does not occur in 4'-epidoxorubicin since the C-4'-hydroxyl is in an equatorial orientation (Cassinelli *et al.*, 1984;

Arcamone *et al.*, 1984). 4'-Epidoxorubicin glucuronide is the major metabolite found in plasma of patients receiving this drug (Robert *et al.*, 1985; Vrignaund *et al.*, 1985), and this glucoronidation is believed to underlie the enhanced rates of elimination of 4'-epidoxorubicin compared with doxorubicin (Weenen *et al.*, 1984). Dextoxification of quinones by conjugation to glucuronic acid is facilitated by their two-electron reduction (Lind *et al.*, 1990).

In considering the EO9 analogues, improvements in the plasma half-life might be achieved through modelling structural changes to the quinoid nucleus based upon observations of the superior plasma stability of MMC. In humans, the plasma pharmacokinetics of MMC elimination has a $t_{½}$ = 50 minutes (Verwey *et al.*, 1987), while $t_{½}$ value for EO9 was 7.8 ± 5.6 minutes (range 0.8-19 min) (Schellens *et al.*, 1994). Similarly, a very short plasma half-life was seen for EO9 in murine studies ($t_{½}$ =1.9 minutes) while the $t_{½}$ for MMC was 8-fold longer (Workman *et al.*, 1992b) The poor pharmacokinetics of EO9 is thought to account, in part, for the poor performance of this agent in phase II clinical trials (Wanders *et al.*, 1995; Pavlidis *et al.*, 1996; Dirix *et al.*, 1996), and has raised doubts as to whether the initial clinical scheduling is optimal (5 minute infusions or bolus injections) (Pavlidis *et al.*, 1996; Smitskamp-Wilms *et al.*, 1996). Alterations to the quinoid nucleus of EO9 which mimic the structural details of MMC, such as the methylation of the C-6 position, could provide agents with superior pharmacokinetic properties. It must also be considered that the clinical efficacy of MMC might also be a consequence of the relatively slow rate of pharmacodynamic metabolism of MMC by various flavoproteins (E_7^1 = -310 mV), which will contribute towards effective tissue penetration. Modification of EO9 at the C-5 position (i.e EO8 & EO7) lowers the E^1 of these agents to similar values reported for MMC, and may thus improve tissue penetration properties.

7.7 Future prospects of hypoxia-activated cytotoxins

The utility of hypoxia-specific cytotoxins has not been conclusively demonstrated in the clinic, with neither CI 1010 nor EO9 having so far lived upto expectations. The randomised phase III clinical trials evaluating the impact of TPZ in combination with conventional anti-tumour therapies are eagerly awaited. TPZ would not be normally considered as an appropriate prodrug to utilise in combination with an oxygen-sensitive GDEPT approach, since its active metabolite is too labile to produce any direct bystander effects. Yet, one might envision that replacing the membrane anchor of P450R with a trans-cellular targeting sequence could ensure export from a transduced cell and import into

neighbouring cells. Thus rather than relying on activated metabolite distribution, novel approaches aimed at distributing the enzyme throughout the target tissue would increase the bystander effect with clinically well advanced agents such as TPZ. Such export/import targeting signals might be obtained from a diverse array of biological systems including; basic fibroblast growth factor (Sosnowski *et al.*, 1996), the Drosophila antennapoedia protein (Derossi *et al.*, 1994), the VP22 protein from herpes simplex virus (Phelan *et al.*, 1998), truncated Pseudomonas aeruginosa exotoxin A encoding the translocating domain (Chen *et al.*, 1997) or simple membrane-translocating carrier peptides (Rojas *et al.*, 1998). These protein fusions could be further refined by including nuclear localisation sequences to target TPZ activation to the nuclear compartment (Nigg, 1997). This approach has proved sucessful with nuclear-localised P450R in combination with MMC (Belcourt *et al.*, 1998). These concepts are the subject of ongoing studies.

7.8 Amplifying transcriptional output in response to hypoxia.

A desirable property in any GDEPT strategy is a large output of therapeutic gene message and protein product in response to an appropriate cellular environment. Many mechanisms can influence the expression of a gene, including chromatin structure, transcription, transcription termination, RNA processing, and mRNA stability. Of these, transcription is perhaps the primary mechanism by which oxygen regulated gene expression is modulated in response to hypoxia. An important characteristc of transcription factors is that they are modular, such that one module can make sequence specific contacts with DNA, while other modules are required to interact with the basal transcriptional machinery, with intermediary co-activator proteins or with other adjac ent bound transcriptionfactors (Pabo and Sauer, 1992). Genes respond in a unique fashion to enviromental and cell-specific signals, and regulation is governed, in part, by the specific array of transcription factor binding sites.

HIF-1 binding sites are no exception, with sequence conservation existing not only at HIF-1 binding sites, but also at precisely distanced adjacent sites shown to be of functional relevance, suggesting that the factors which bind at these adjacent sites are important for both the operation of HIF-1, and the cooperative induction of gene expression in response to low oxygen tension. Adjacent transcription factors bound to promoters and enhancers probably form stereospecific nucleoprotein complexes which are capable of inte grating responses from multiple signal transduction pathways (Tjian and Maniatis, 1994). For example, in the LDH-A promoter, the HIF-1 site interacts with a cAMP-response element (CRE) located 14 bp. away, and both elements are essential for enhancer activity, and the LDH-A HIF-1 element is not

capable of independent function (Firth *et al.*, 1995). Similarly, the Epo 3' enhancer is functionally dependent on a putative steroid response element which interacts with various orphan nuclear receptors (Blanchard *et al.*, 1992; Galson *et al.*, 1995). A synergistic interaction has been observed between the serum-response element (SRE) and the HIF-1 site in the murine GLUT-1 5' enhancer which is located at -3.3 to -2.7 kb from the transcription start site (Ebert *et al.*, 1995a). Also, the co-activators p300/CBP have been demonstrated to interact with the C-terminus of HIF-1α (Arany *et al.*, 1996), the potential role of which is to serve as a mediator of 'cross-coupling' between HIF-1α and other transcription factors, perhaps forming an activation scaffold (Chakravarti *et al.*, 1996). P300 and/or CBP also co-activate the cAMP-responsive binding protein (CREB), and may mediate the cooperative interaction between the CRE and HRE in the LDH-A enhancer.

Further HIF-1β (when dimerised with AhR) has been shown to interact directly with the zinc finger domain of the proximal promoter factor SP1 *via* its bHLH and PAS domains. The binding of either transcription factor to its cognate DNA element facilitated the binding of the second factor, inducing a synergistic effect on CYP1A1 gene promoter activity (Kobayashi *et al.*, 1996). This observation likely extends to HIF-1α/β heterodimers, and it is of note that HRE and GC box elements have been found in proximity in several hypoxia-responsive gene promoters, including VEGF and GLUT-1 (Levy *et al.*, 1995; Ebert *et al.*, 1995a). This suggests a mechanism by which HIF-1α/β may directly interact with the basal transcriptional apparatus assembled in proximal promoter regions, and might even play a role in the transcriptional synergy that is seen between distally and proximally bound SP1 (Courey *et al.*, 1989). These observations imply a possible mechanism by which long distance interactions may be encouraged between HIF-1/SP1 bound enhancers and their proximal promoter regions. Interestingly, the VEGF minimal promoter, which lacks its HIF-1 binding site(s) but contains 3 SP1 sites, retains some hypoxia-inducible properties (Levy *et al.*, 1995), which may reflect the redox-sensitivity of SP1 *via* its zinc finger sulphydryl groups (Wu *et al.*, 1996; Ammendola *et al.*, 1994; Knoepfel *et al.*, 1994). The rational design of HRE-driven therapeutic DNA constructs to incorporate the potential for cooperative interactions between HIF-1 with other constitutive or inducible transcription factors, represents one method by which large transcriptional outputs might be achieved in response to hypoxia.

The marked synergy observed between hypoxia and phorbol 12-myristate 13-acetate (PMA) in the GLUT-1 5' enhancer, is an example of cooperativity between distinct elements. The exploitation of GLUT-1 regulatory sequences is attractive in view of its well documented upregulation in many neoplasias (Younes *et al.*, 1996; Mellanen *et al.*, 1994; Glick *et al.*,

1993; Minn *et al.*, 1991; Yamamoto *et al.*, 1990). Deletion analysis has demonstrated that both functional HIF-1 and SRE/TRE binding sites are essential to produce an augmented response (Ebert *et al.*, 1995a). Phorbol esters can substitute for diacylglycerol (DAG) in the activation of the serine/threonine kinase PKC, but unlike DAG they are not rapidly metabolised and are therefore useful for *in vitro* analysis of PKC-dependent signal transduction. PKC in mammals is comprised of at least 12 distinct polypeptides or isoforms that orchestrate multiple cellular functions including differentiation, growth and transcription (Review: Dekker and Parker, 1994). PKC-mediated signal transduction through the activation of mitogen-activated protein kinases (MAP kinases) stimulates several transcription factors that interact with and activate the SRE (Treisman, 1992). SRE activity is also stimulated by activated forms of membrane-associated tyrosine kinases such as Src, and by activated forms of c-Raf and Ras, signal pathways that also stimulate HIF-1. The serum response factor (SRF) binds directly to the SRE and forms a complex with a ternary complex factor (p62/TCF), which can be composed of any member of the Ets domain proteins, incuding SAP-1, SAP-2, Elk-1 and NET. SRF is directly implicated in the regulation of SRE activity, and at least one of its accessory proteins, Elk-1, appears to be a direct target for phosphorylation by MAP/ERK kinases. Other transcription factors including NF-IL6 and AP1/ATF can also bind the core SRE. The PKC-dependent MAP kinase cascade also stimulates the gene products of the proto-oncogenes Fos and Jun which specifically bind to AP-1 (TRE) sites. It is therefore of note that the GLUT-1 SRE has adjacent to AP-1/TRE binding sites, which are juxtaposed to the core SRE motif. (Ebert *et al.*, 1995a). The SRE is induced by a variety of factors, including serum, mitogens, H_2O_2, UV-A radiation, cAMP and mitochondrial inhibitors (Treisman, 1994; Ebert *et al.*, 1995a).

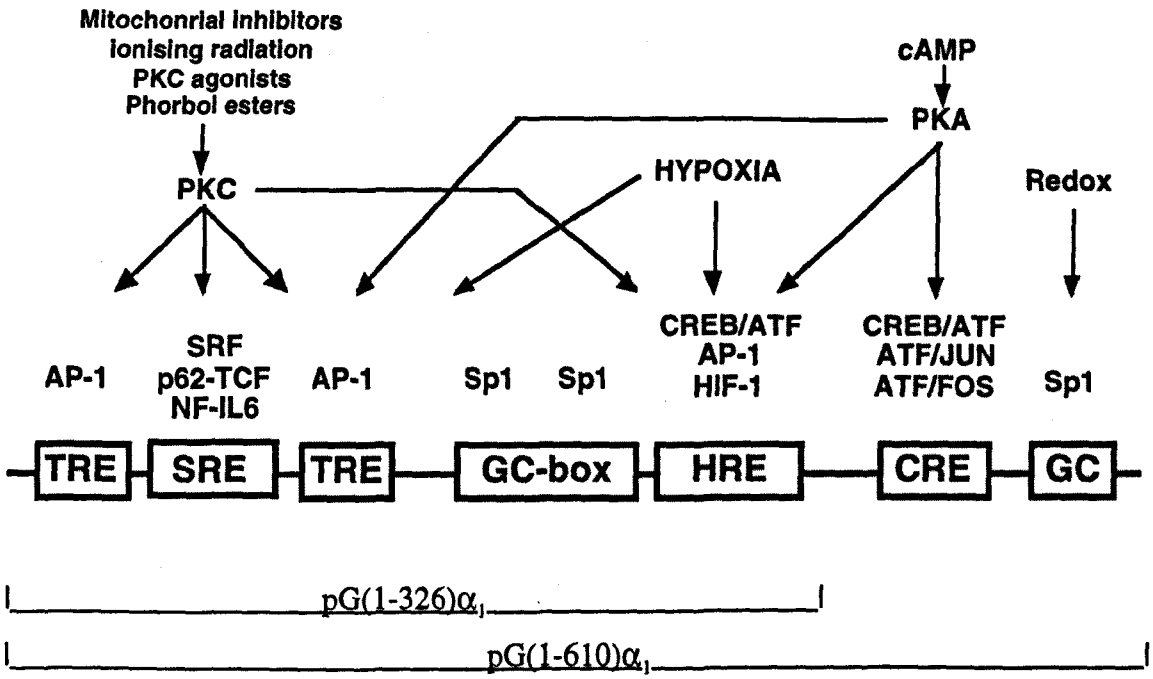
Since mitogens such as PMA are tumour promoters, other pharmagological agents must be considered as co-stimuli to induce the transcriptional synergy observed in the GLUT-1 enhancer *in vivo*. It may beadvantageousif potential agents have independent anti-neoplastic properties. Candidates might include the PKC partial agonist, bryostatin-1 (Zhang *et al.*, 1996), the mitochondrial inhibitor, MKT-077 (Koya *et al.*, 1996), the PKA modulator, 8-Cl-cAMP (and 8-Br-cAMP) (Cho-Chung, 1990), or perhaps γ-irradiation. The macrocyclic lactone bryostatin-1 modulates PKC activity by binding and activating the enzyme, but in the presence of activating ligands it will behave primarily as an antagonist. It has currently completed three phase I clinical trials and some antitumour activity has been reported. The positively charged rhodacyanine dye, MKT-077, selectively accumulates in the mitochondria of neoplastic cells due to the elevated negative transmembrane potentials that are characteristic of the transformed state. This might allow the targeted inhibition of mitochondrial function in

tumour tissues, possibly providing an additional level of specificity in the regulation of the induction of the GLUT-1 enhancer-regulated therapeutic construct. Alternatively, induction of cAMP-dependent PKA type II activity with the cAMP analogues, 8-Cl-cAMP and 8-Br-cAMP, may stimulate the SRE and AP-1/TRE sites in a similar fashion to that seen with PKC-dependent activation. This is suggested by evidence implicating PKA in the nuclear localisation of the transcription factor NF-IL6, which binds to the SRE in the c-fos promoter in response to cAMP stimulation by forskolin (Metz and Ziff, 1991). This re-distribution of NF-IL6 from the cytoplasm to the nuclear compartment correlates with increased phosphorylation of NF-IL6 suggesting its involvement in SRE regulation by cAMP. Further, PKA activation promotes the nuclear entry of SRF, although this appears not to be directly dependent on SRF phosphorylation, since a putative PKA phosphorylation site on SRF (serine-103) is not essential for nuclear localisation. Nevertheless, 8-Br-cAMP or microinjection of the C subunit of PKA initiates the redistribution of SRF to the nuclear compartment. Nuclear localisation could be specifically inhibited by microinjection of a specific inhibitory peptide against PKA (Gauthier-Rouviére *et al.*, 1995). Also of potential relevance, PKA-mediated phosphorylation is suggested to be invol ved in the nuclear translocation of c-fos (Roux *et al.*, 1990), and the AP-1 enhancer element can be regulated by PKA activation (Merino *et al.*, 1989). Fos and Jun can also form heterodimers with CREB to activate genes with either the AP-1/TRE or CRE elements (Hai and Curran, 1991), both of which are present in the GLUT-1 5'-enhancer.

Although PKA and PKC are independent signal transduction pathways, there is evidence that "cross-talk" occurs between the two pathways, implying that if one pathway is induced or repressed, the other may be affected. For example PKC, but not PKA, stimulates the formation of transcriptionally active CREB dimers in PC12 cells, a process that is dependent upon CREB phosphorylation (Yamamoto *et al.*, 1988). PKA stimulates CRE-dependent transcription without influencing dimerisation, suggesting dual regulation of CREB binding activity and transcriptional efficacy. Also cAMP has been shown to stimulate the translocation of PKC to the nuclei of B lymphocytes (Cambier *et al.*, 1987).

In addition to the therapeutic co-stimuli, several other agents were employed in the initial assessment to determine which cellular stimuli were relevent to the augmentation of GLUT-1 enhancer activity. These included the PKC inhibitor, calphostin C, and two inhibitors of the mitochondrial electron transport chain, rotenone and azide.

These potential signal transduction pathways and their possible sites of action on the multiple transcription factors that bind and activate the GLUT-1 enhancer are illustrated in figure 1. Signal integration might also involve co-activators such as p300/CBP, also known targets of the action of cAMP-dependent protein kinase A.



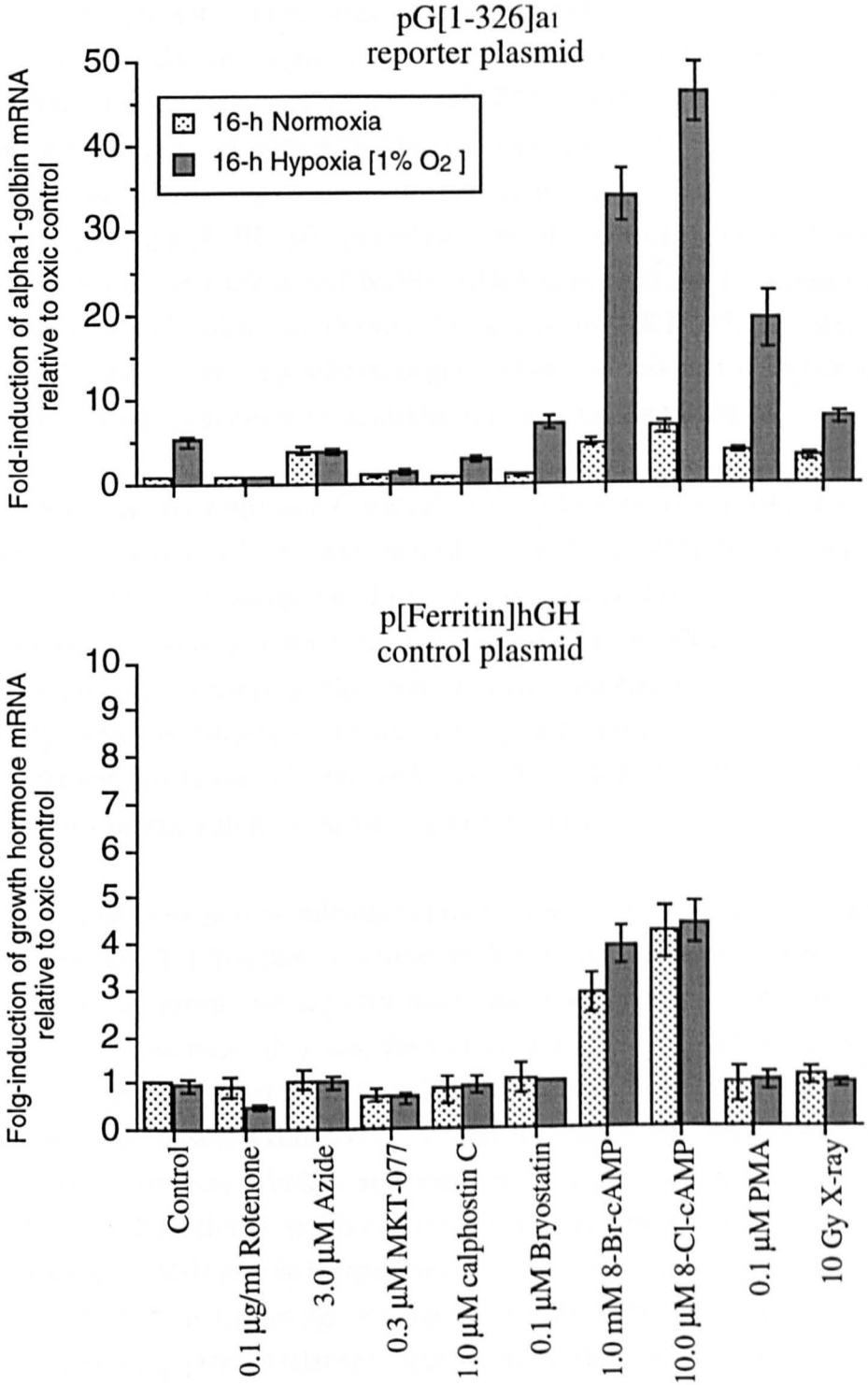
Preliminary experiments have been conducted, utilising the transient transfection of the short 1-326 bp. GLUT-1 enhancer fragment in order to identify which clinically relevant co-stimuli might mimic the synergistic effects of phorbol esters on GLUT-1 5'-enhancer activation when combined with hypoxia. Ebert *et al.* (1995a) identified the shortened pG(1-326) GLUT-1 sequence as sufficient to confer an augmented response to both PMA and hypoxia. This enhancer fragment lacks the 3' CRE and Sp1 sites, but was found to be modestly more responsive to hypoxia compared to the complete pG(1-610) sequence (6 *vs.* 4-fold). pG(1-326) also exhibited lower constitutive activity under normoxia (63% of full enhancer). A single copy of the truncated 1-326 bp. enhancer sequences, in the context of the short α_1-globin promoter, was employed to regulate α_1-globin mRNA. Transcriptional activity of these reporter constructs was monitored by RNase protection assay as described by Ebert *et al.* (1995a) and standardised against an internal transfection control reporter construct (ferritin promoter-driven human growth hormone (hGH)). The human breast adenocarcinoma cell line, MDA 468 was used to examine the influence of 16-h hypoxia (1% O_2) ± co-stimuli,

following transfection of the reporter plasmids. Following RNA extraction, mRNA levels for both α_1-globin and hGH were determined by cross-hybridization of appropriate ^{32}P-labelled riboprobes, followed by quantification of the corresponding protected mRNA bands using a flat-bed scintillation counter. The exact details of reporter plasmids, mRNA extraction, riboprobes and experimental techniques have been described by Ebert *et al.* (1995).

Transient transfections are non-physiological in several respects, including the abnormal copy number of reporter genes per cell, unchromatinised templates, and the time-course of expression of transiently transfected plasmids. Probably of most significance, chromatin serves important roles in regulating gene expression, by controlling access to the DNA template and bringing regulatory sequences which are widely separated into close contact, enabling the interaction of *trans*-acting factors bound to dispersed *cis*-acting sequences (Wolffe, 1994). The patterns of nucleosome binding are dynamic, changing with alterations in gene expression (Lewin, 1994). Nevertheless, transiently transfection reporter gene-readout experiments represent a valuble experimental tool for studying enhancer functions and the potential interactions of transcription factors, even though they may not accurately describe the response of the native sequences. The therapeutic relevance of these differences will depend upon the method of gene delivery employed to transduce the target tissues.

The results of this preliminary analysis were quite striking. Transcriptional synergy induced by hypoxia (1% O_2) and PMA co-treatment was seen, with similar values ($\approx$ 18-fold) to that reported by Ebert *et al.* (1995a). The type II PKA upregulators 8-Cl-cAMP and 8-Br-cAMP markedly synergised with hypoxia in the activation of the pG[1-326]α_1 reporter plasmid, generating upto 47-fold induction of α_1-globin mRNA. The superior effects of these cAMP analogues relative to PMA may be a reflection of their multiple influences on HIF-1, SRF, CREB, NF-IL6 and AP-1, any combination of which might facilitate the observed transcriptional interactions. Modest stimulation of the reporter plasmid by the cAMP analogues was also apparent under aerobic conditions ($\approx$ 5-fold), which was approximately similar to the effect of 1% O_2 treatment alone. In addition, both cAMP analogues modestly stimulated the constitutive ferritin promoter-regulated control plasmid, suggesting that modulation of PKA II can have multiple effects on gene expression.

Figure 2: Regulation of the truncated GLUT-1 enhancer, pG[1-326]α_1, by various PKC, PKA and SRF stimuli, with or without co-treatment under hypoxia (1% O_2) in the human breast adenocarcinoma cell line MDA 468.

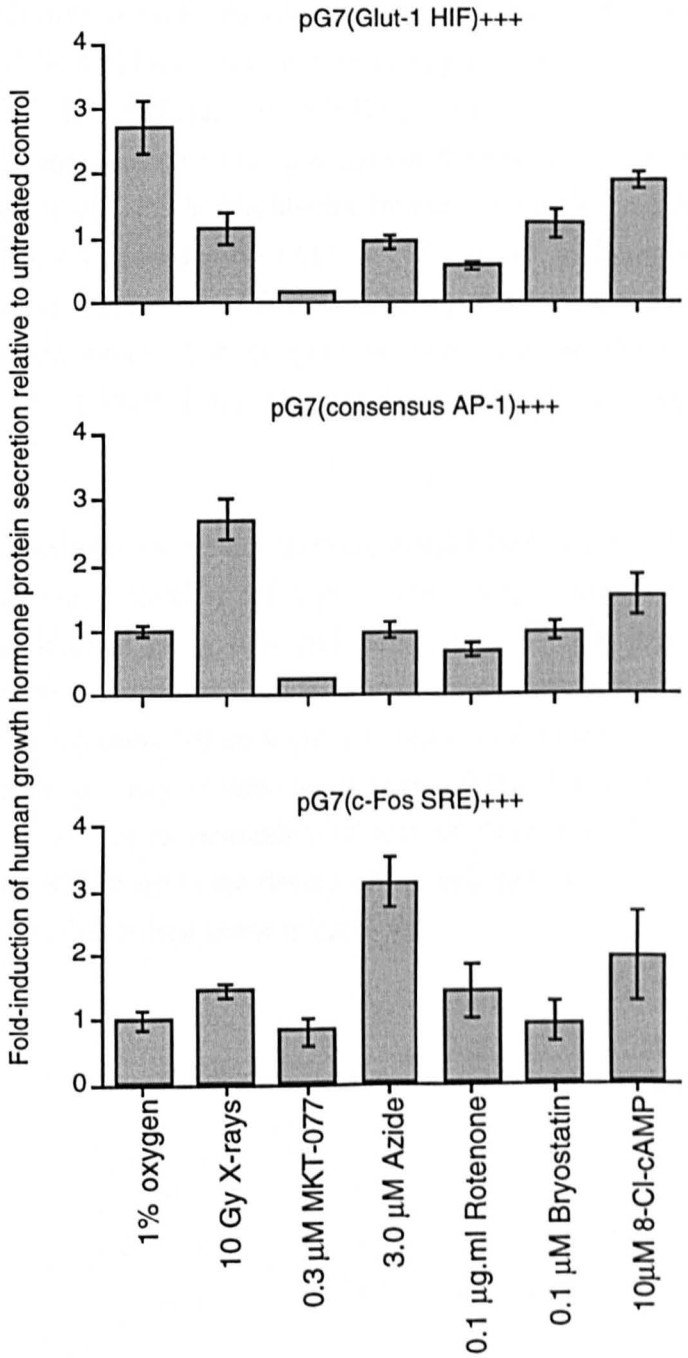


The mitochondrial inhibitors rotenone and azide could not mimic the augmented response of the GLUT-1 enhancer to the phorbol ester and cAMP analogues, despite their known stimulatory influence on the activity of the SRE (Ebert *et al.*, 1995a). Azide stimulated GLUT-1 dependent reporter gene activity under normoxic conditions, but co-stimulation with hypoxia was apparently not additive. This is in contrast to a recent report, where similar co-stimulation was reported to be additive with respect to GLUT-1 protein expression (Behrooz and Ismail-Beigi, 1997). Both rotenone and MKT-077 strongly inhibited α_1-globin mRNA expression which could be overcome by hypoxic stimulation. This perhaps suggests some level of inteference in the energy requirements for transcription and/or mRNA processing. Fluorescence microscopy of MKT-077 treated cells revealed a strong accumulation of the dye in both mitochondria and nucleosomal bodies, which contrasted with the mitochondrial-specific uptake of rhodamine 123 (data not shown). In the case of MKT-077, the observation of highly localised intra-nuclear accumulation might explain the observed inhibition of mRNA expression, and perhaps suggests a novel mechanism of action for this agent.

The PKC inhibitor calphostin C partially inhibited the hypoxic response in the MDA 468 cell line, which is consistent with published reports in other transformed cell lines (Salceda *et al.*, 1997). However the PKC partial agonist bryostatin-1 only modestly stimulated the GLUT-1 enhancer at a concentraton of 100 nM, the effects of which were only additive in the presence of hypoxia. However, at higher concentrations (1 μM) bryostatin-1 was inhibitory, which is consistent with its mixed agonist and antagonistic effects on PKC activation. Ionising irradiation (10 Gy) could also stimulate pG[1-326]α_1 reporter plasmid activity, and co-treatment with hypoxia was apparently additive.

In an attempt to localise the influence of these stimuli to the independent subsequences found within the GLUT-1 5'-enhancer, a series of further transient transfection reporter gene experiments were performed, utilising oligonucleotides corresponding to the GLUT-1 HRE, the c-*fos* SRE or a consensus AP-1 site. Each element was concatemerised as a triplet and cloned 10 bp 5' to the TATA box in a minimal thymidine kinase promoter linked to a human growth hormone (hGH) readout (Ebert *et al.*, 1995a). The MDA 468 cell line was transfected with these reporter constructs as before, and pooled transfections were divided and cells were exposed to the individual stimuli. In this experiment, reporter gene readout was by means of a radioimmunoassay for hGH protein secreted into the media over a period of 96-h (hGH assay performed by Dr. Gillian Campling, Warneford hospital, Oxford). The levels of hGH secretion (mU/L) were expressed relative to a untreated control for each reporter plasmid.

Figure 3: Relative induction of pTKGH reporter plasmids under the transcriptional control of either GLUT-1 HRE, c-fos SRE or consensus AP-1 triplet sites. Cells were exposed to stimuli for 16-h (except x-rays), washed free of the agent, and conditioned media was harvested after 96-h. Data are the mean of two independent experiments.



GLUT-1 HIF-1 activation was stimulated by 1% O_2 and 10 μM 8-Cl-cAMP. A very modest stimulation was seen for 100 nM bryostatin-1, but this was not apparent at other concentrations. No other agents were stimulatory, although MKT-077 strongly inhibited hGH production. The consensus AP-1 triplet was induced by γ-irradiation (10 Gy), and modestly by 8-Cl-cAMP. The mitochondrial inhibitors azide and rotenone were ineffective, although like the HIF-1 reporter, MKT-077 was strongly inhibitory. The c-*fos* SRE driven reporter plasmid was reproducibly induced by azide, rotenone, 8-Cl-cAMP, and γ-irradiation. The suppression of hGH by MKT-077 was not seen, suggesting that perhaps SRE stimulation was compensating for the interference with hGH production. Together, these results imply that the GLUT-1 5' enhancer conveys transcriptional responses to hypoxia, mitochondrial inhibitors, γ-irradiation and PKA modulators through individual elements. The increased activation of the GLUT-1 enhancer by PMA or 8-Cl-cAMP in combination with hypoxia resulted from a positive integration of distinct inducible responses, which exert influence on different cis-acting sequences. The cooperative behaviour of these elements could be harnessed in the design of future therapeutic constructs aimed at exploiting tumour hypoxia in a GDEPT strategy.

Many other mechanisms besides transcriptional initiation can potentially influence the level of gene expression in specific cell types under precise enviromental conditions. For example, both transcriptional elongation and termination are possible sites of regulation (Greenblatt *et al.*, 1993), and mRNA must undergo many stages of processing including 5' capping and 3' polyadenylation before it can be translocated to the cytoplasm (Rosbash and Singer, 1993). In principle, any of these steps could be rate limiting for the production of a therapeutic mRNA. A greater understanding of each of these individual processes and their interacting influences will facilitate the design of optimal therapeutic gene constructs that are efficiently expressed in the desired target tissues.

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